Genetic studies on sex determination and colouration in Nile tilapia (*Oreochromis niloticus* L.)

A Thesis Presented for the Degree of "DOCTOR OF PHILOSOPHY" to University of Stirling

by

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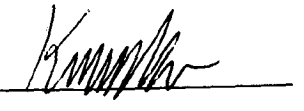
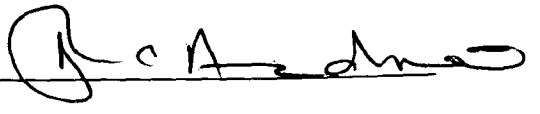
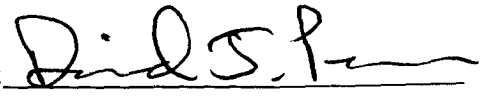
DECLARATION

I hereby declare that this thesis was composed by myself and is the result of my own research. It has neither been accepted nor submitted for any other degrees. All the sources of information have been duly acknowledged.

Signature of candidate 

Signature of supervisor 

Signature of supervisor: 

Date: 6/8/99

DEDICATED

TO

MY MOTHER, AYSEL

MY FATHER, AHMET

MY HUSBAND, SEDAT

MY SON, ANIL

FOR THEIR ENDLESS LOVE AND SUPPORT

ABSTRACT

The present study was undertaken to investigate colour and sex determination mechanisms through the application of androgenesis, gynogenesis and controlled breeding programme with the objective of producing all red males in *O. niloticus*.

The highest yield of androgenetic haploid to pigmentation stage was 24.6±3.5% (relative to controls) with optimal UV irradiation dose of $450Jm^{-2}$ for 5 minutes. The highest survival rate of diploid androgens was 0.07±0.07% (relative to controls) to yolk sac stage using a heat shock of 42.5°C for 3 minutes 30 seconds applied at 25 minutes after fertilisation. All paternal inheritance of diploid androgenetic tilapia was verified using DNA fingerprinting.

The mean recombination frequency of the red skin colour gene in meiotic gynogens was 0.12±0.04. All maternal inheritance of meiotic gynogens was verified using the isozyme locus ADA*. Analyses of sex ratios of meiotic gynogens suggested that male progenies were produced by an epistatic sex determining locus (SDL-2 with two alleles SR and sr) causing female to male sex reversal in the homozygous phase (srsr) but with limited penetrance. A close linkage was found between a sex determining locus (SDL-2) and the red gene.

No significant difference was found between colour genotypes (namely homozygous red, heterozygous red and wild type) in terms of total fecundity, ISI (inter spawning interval), egg size and survival rate. Overall mean ISI was 26.3±1.0 days. Mean total fecundity was 1096 eggs. Fecundity varied over successive spawns but this variation did not appear to be related to spawning periodicity.

Hormonal and thermal feminisation were compared on all YY male progeny of *O. niloticus*. While similar female percentages of 32.0±5.2 and 33.8±1.5% were produced,

significantly higher intersex percentages of 18.5±2.5 and 1.6±0.8 were observed in heat and DES treated groups, respectively. Heat treatment groups showed the lowest survival rate of 62.6±9.8% compared to the survival rates of 97.0±0.9% and 97.3±0.8% in controls and DES treated groups, respectively.

YYRR males and YYRR neofemales were produced by integrating existing YYrr males and YYrr neofemales from the Egypt-Swansea-Philippine isolate and YYRR androgenetic males from the Stirling isolate with XXRR females and XYRR males of the Stirling isolate of Egyptian strain *O. niloticus*.

In summary, this study provides valuable information regarding the colour and sex determination mechanisms of *O. niloticus*. The research in this thesis also demonstrated that both YY genotype and red coloration can be combined in a single strain in order to produce all male and stable red coloured *O. niloticus*.

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Chapter 1:

General introduction

1. General Introduction

1.1. Introduction

Statistics show that the average per capita daily calorie intake of most people who live in lesser developed countries comes to about 70% of that in the more developed world. Hundreds of millions of people consume quantities of protein each day which are below the standard requirement for normal growth and development. Thus, many food production programmes have tended to concentrate on any potential source of cheap, abundant and palatable protein. Fish is one of the most important sources of animal protein. It is preferred in some respect over other animal proteins because of special qualities like low fat content and highly unsaturated fatty acids that are beneficial for human health.

1.2. Tilapia

Tilapia is the common name for over 70 fish species belonging to the Tribe Tilapiini, an African group of fish within the Family *Cichlidae*. Previously regarded as members of a single genus, *Tilapia*. Today, three main genera are now generally recognised based on taxonomic revision considering differences in feeding habits, biogeographical distribution but principally mode of reproduction (Trewavas, 1983). These are *Tilapia*, substrate spawners and guarders, *Sarotherodon*, paternal or biparental mouthbrooders, *Oreochromis*, maternal mouthbrooders (Trewavas, 1983).

Huet (1970) quoted 16 species while Balarin and Hatton (1979) listed 23 species, which had been subjected to either experimental or commercial culture. Realistically, only eight or nine species of tilapia have significant potential in aquaculture (Schoenen,

1982; Pullin, 1983). Among them, only *Tilapia* (*T. zillii* and *T. rendalli*) and *Oreochromis* (*O. niloticus*, *O. mossambicus*, *O. hornorum* and *O. aureus*) species are in widespread use (Hepher and Pruginin, 1982). Of these species, the Nile tilapia, *O. niloticus*, is by the far most important in freshwater culture regimes (Table 1.1.; Macintosh and Little, 1995).

Table1. 1. Reproductive characteristics of the tilapiine genera and the main species of importance in aquaculture (Source: Macintosh and Little, 1995).

Genus	Mode of reproduction	Important species in aquaculture
Tilapia	Substrate-spawners	*T. zillii*
	(guarded nests)	*T. rendalli*
Sarotherodon	Paternal or bi-parental	*S. galilaeus*
	mouthbrooders	
Oreochromis	maternal mouthbrooders	*O. niloticus* *O. mossambicus* *O. aureus* *O. urolepis-hornorum* *O. andersoni* *O. macrochir* *O. spilurus*

Over recent years tilapia have steadily grown to become one of the most commercially important groups of freshwater fish species in tropical aquaculture. Although tilapia are endemic to African countries, interest in tilapia culture potential has led to their distribution to over 100 tropical and sub-tropical countries.

Tilapias are of great importance and represent an ideal warm water food fish, being available from wild sources and suitable for a wide range of aquaculture systems from

simple small-scale waste-fed fishponds to intensive culture systems. They have been labelled as the "aquatic chicken" because of their farmed profitability in a wide range of systems (Pullin, 1996).

Tilapias are widely recognised as the most suitable species for aquaculture due to several beneficial physiological attributes. Firstly, tilapias can tolerate a variety of environmental conditions including high salinity, low oxygen tension and overcrowding. The high growth rate of tilapia, even with the use of natural and cheap artificial food is one of the other advantages for aquaculture. Tilapias have relatively short reproductive cycles, breed prolifically under culture conditions, are strongly resistant to disease and are amenable to handling and captivity (Balarin, 1979). In addition, they posses good characteristics as a table fish with a low level of fat in the flesh, no intramuscular bones, a fine taste and fillet yield of 32% in fish with an average weight of 0.45 kg. Table 1.2. shows the most widely cultured tilapia species and hybrids with their current and potential importance in aquaculture.

1.3. Tilapia aquaculture

Tilapia culture is believed to have started some 4000 years ago in Africa, 1000 years before carp culture was first started in China but the first scientifically orientated culture of tilapia was conducted in Kenya in 1924 (Balarin and Hatton, 1979). The proper cultivation of this fish began during and after the Second World War in the 1940's. There is already a significant tilapia culture in China, Indonesia, Philippines, Sri Lanka, Thailand, Vietnam and Bangladesh. Interest in tilapia culture is also increasing elsewhere in the Americas, Venezuela, Colombia, and the USA (Pullin, 1983, 1996; Eknath *et al.*, 1991). According to Pullin (1996) tilapia are now being farmed or researched in 71

Table 1. 2. Tilapias of current or potential importance in aquaculture (Source: Pullin 1983, 1991; Lowe-McConnell, 1988: cited in Pullin, 1991)

Species	**Evaluation**
O. niloticus	Fast growth, especially in the tropics; versatile feeder.
O. aureus	Fast growth; versatile feeder; tolerant of cold but difficult to seine in ponds, so best grown in cages or used as a male parental stock for hybridisation.
Monosex male hybrids: *O. niloticus* X *O. aureus*	Fast growth, especially on pelleted feeds.
T. rendalli	Macrophyte feeder; potential for polyculture with microphagous tilapias; excellent keeping qualities.
O. spilurus spilurus	Fast growth; tolerant of salinity; good grazer on epiphtic algae.
O. andersoni	Reasonable growth and tolerance of cold.
S. melanotheron	Tolerant of salinity; good growth in experimental separate-sex culture.
Red tilapias-a group of single species (principally *O. niloticus* and *O. mossambicus*) or more often hybrids of these and/or *O. aureus* and/*or O. urolepis hornorum*	Mixed information, but generally good growth and sometimes preferred to normal-coloured fish in markets: some hybrids do well in seawater cage culture(e.g. *O. mossambicus*/*O. urolepis hornorum* hybrids)

countries including developed nations where tilapia are becoming popular with consumers (Stickney, 1994). Despite the early spread of tilapias to over 100 countries (Balarin and Hatton, 1979) and their many suitable qualities, the overall world production of tilapia (principally *O. niloticus*) is 5.3% (800,823 mt) of the total freshwater fish and shellfish production of 15,082,225 mt. Carp (many species) is still dominant with 76.3%

or 11,504,352 mt (FAO, 1998). Although they are African fish, China, Philippines, Taiwan, and Thailand are the world's leading tilapia producers (Fig. 1.1; Macintosh and Little, 1995). Although there are many positive attributes of tilapia as farmed fish, the constraints to expansion of tilapia culture are negative attitudes and policies such as poor breeds, early maturation, poor growth, colour, poor non-sustainable farming systems and possible adverse environmental impacts (Pullin, 1996).

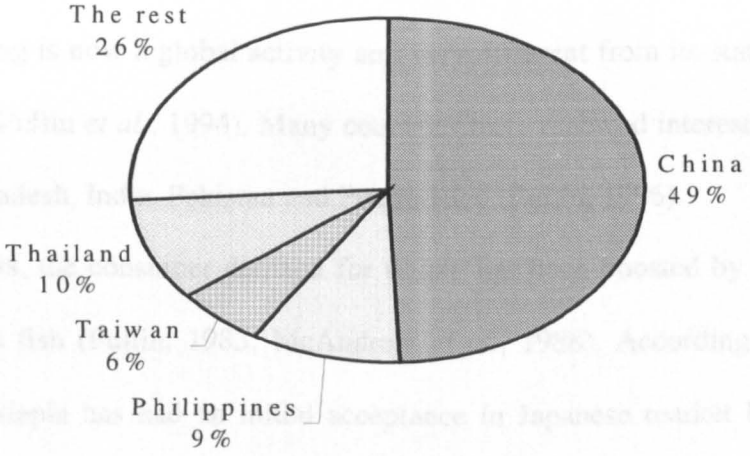


Figure 1. 1. Tilapia production of the world leading producers and the rest of the World (Data from FAO, 1998).

In many African countries, small-scale tilapia farming is in progress (de Kartzow, 1992) but intensive tilapia culture in tanks and raceways is also now operating commercially in Zimbabwe (Madhu, 1992).

In Asia, especially in Thailand and Philippines, *O. niloticus* is being reared in small ponds on agricultural wastes, under integrated aquaculture systems (Edwards, 1983; Guerrero, 1987; Edwards *et al.*, 1988). The use of domestic wastewater to rear carps and tilapia in Vietnam and their integration with rice and/or vegetable production has been reported by Pham and Vo (1990).

Intensive pond, raceway, and tank culture systems to produce *O. niloticus* or its hybrids are operated on a commercial basis globally in Taiwan (Chen, 1990), Florida (Sipe, 1992), Zimbabwe (Madhu, 1992), Malaysia and Costa Rica (Macintosh, 1993). Table 1.3. shows the problem associated with tilapia farming system.

Tilapia farming is now a global activity and very different from its status as recently as 10 years ago (Pullin *et al.*, 1994). Many countries have renewed interests in culture of tilapia e.g. Bangladesh, India, Pakistan and Puerto Rico (Pullin, 1996).

In recent years, the consumer demand for tilapia has been boosted by emergence of red forms of this fish (Pullin, 1983; McAndrew *et al.*, 1988). According to Fitzgerald (1979), the red tilapia has had an initial acceptance in Japanese market because of its similarity to the popular sea bream, *Chysophyrs major*, and its colourless mesentery which makes it attractive for the preparation of traditional "Sashimi". Red tilapia are commercially cultured in Taiwan (Kuo, 1988), the Philippines (Radan, 1979), Israel (Banash, 1984; Berger and Rothbard, 1987), USA (Sipe, 1992), Greece (Anon, 1984), Malaysia (Macintosh and Little, 1995) and sold in supermarkets such as TESCO in U.K. (personal observation).

Despite their remarkable qualities, tilapias are notorious due to their habit of prolific breeding. In the tropics, tilapia can become sexually mature and begin to reproduce at an

Table.1. 3. Problems associated with tilapia farming systems (Source: Pullin, 1996).

System	Major problems	Farmers' need
Cages	*Ad hoc* design, guessed at or copied from elsewhere; poor feed conversion; fouling; short operational life.	System specifically designed for tilapias in fresh-, brackish- and saltwater.
Pens, acadja-enclos, etc.	Still experimental.	Reliable, sustainable systems that match their resources.
Ponds	Nutrient starvation; *ad hoc* stock management; water availability/quality.	Sustainable systems, well-integrated with other enterprises.
Tanks, raceways and other intensive systems, including recycling	Largely experimental or guesswork at site-specific designs.	Reliable guidelines-as exist for trout culture.
Hatchery/nursery systems	Low and/or seasonal output of fry/fingerlings; no consideration of genetic consequences of broodstock management; low adoption of monosex and seed technology.	Reliable seed supply systems that maintain genetic quality and 100% male seed production, where such is appropriate.

early stage in their development, when about four to five months of age or even younger (Wohlfarth and Hulata, 1983; Tave, 1990; Swift, 1993) and at a size as small as 6 to 15 cm (Babiker and Ibrahim, 1979; Dadzie and Wangila, 1980; Tave, 1990; Swift, 1993). The reproductive cycles of females within a breeding group are not synchronised, although each female fish may breed up to 12 times in a year under optimal conditions (Macintosh, 1985). This reproductive efficiency of tilapia results in subsequent unwanted reproduction leading to overpopulation, stunting, unpredictable size of harvested fish and thus the resources (e.g. food, fertilisers, pond, time) will be wasted. One of the simplest method to avoid unwanted production of fry in production ponds is the use of single sex fry populations. The various techniques used to produce single sex, all male, fry will be detailed.

1.4. Monosex male tilapia approaches in aquaculture

Various approaches have been developed to overcome the problems of excessive fry production which cause overpopulation and stunting of cultured population during culture of tilapias which constrains the efficient development and extension of tilapia aquaculture. Evidence shows that males grow larger and quicker than females so interest in single sex, all male culture, gained more attention. The techniques used have changed over time as our understanding of the biology and control of sex in these species has improved. There are mainly two approaches for controlling the reproduction of tilapia: 1) husbandry approaches e.g. using predators, culture in cages and producing monosex populations by manual sexing of fingerlings 2) genetic approaches, e.g. production of sterile fish, interspecific hybridisation, hormonal sex reversal and YY male broodstock

production (Pruginin *et al.*, 1975; Shelton *et al.*, 1981; Scott *et al.*, 1989; Mair and Little, 1991, McAndrew, 1993; Mair *et al.*, 1997). Much research in recent years has concentrated on methods to produce all male tilapia (Hopkins, 1979; Pandian and Varadaraj, 1988, 1990; Mair and Little, 1991; Mair *et al.*, 1993, Mair, 1996).

1.4.1. Manual sexing

Tilapia are unusual among many commercial fish in that they have sexually differentiated genital papillae making it possible to manually sex mixed sex populations (Mires, 1977). Differences between males and females can be seen with the unaided eye at a size of <10 g but for practical purposes the fish are normally sexed at ≥ 30 g (McAndrew, 1993). Manual sexing is not only a labour intensive method with a high risk of human error (Guerrero, 1982; Popma and Green, 1990; Mair and Little, 1991) but it also has the disadvantage of discarding about 50% of the population (i.e. females) although in some countries the latter are processed into fish cakes for human consumption (Akande, 1989) or used as fish and/or animal feed (Wee *et al.*, 1986). Balarin (1982) reported that a skilled worker could sort between 1000-2000 fish per day. However, it is very difficult for even the most skilled workers to achieve greater than 90% accuracy in sexing thus breeding and reproduction is rarely completely controlled.

1.4.2. Predator control

A large number of predators such as *Clarias gariepinus*, *Lates niloticus*, *Bagrus docmac*, *Hemichromis fasciatus*, *Micropterus salmoides* and *Dicentrarchus labrax* have been tried in various part of the world in order to control of tilapia reproduction in ponds.

However, the technique is relatively unspecialised and suited to subsistence aquaculture. The problems in the use of predators involve the lack of full understanding of predator-prey interaction mechanisms, insufficient attention to the selection of an ideal species for use, insufficient data on optimal stocking rates and inadequate appraisal of the ecological interactions in the culture environment (Mair and Little, 1991).

1.4.3. Interspecific hybridisation

The findings by Hickling (1960) of all male fry in crosses between two, of what were believed to be *O. mossambicus* strains, but subsequently were found to be different species *O. mossambicus x O. hornorum* prompted research into finding other interspecific hybrids with the same characteristics (All crosses follow the genetic practise of giving the female parent first). Various theories were then put forward as to possible underlying sex determination mechanisms. These were analogous to the male heterogamety model (XY male and XX female) and the female heterogamety model (ZW female and ZZ male). Whereby crosses between homogametic sexes resulted in single sex offspring (e.g. XX female x ZZ male produces all ZX male). Crosses between *O. mossambicus* and *O. hornorum* consistently producing almost all or all male progeny were also reported by Wohlfarth *et al.* (1983) but slow growth performance and the dark colour of the hybrid constrained the utilisation of this hybrid in commercial production (Wohlfarth, 1994).

Pruginin *et al.* (1975) reported the first hybridisation between *O. niloticus* and *O. hornorum* generating all male progeny and performance of this hybrid was also confirmed by Wohlfarth *et al.* (1983; 1990). The cross *O. niloticus* X *O. hornorum* is not

used commercially due to low and inconsistent fry production, and their unattractive appearance to consumers.

The original cross between *O. niloticus* X *O. aureus* generating all male offspring is utilised commercially by some fish farmers in Israel and Taiwan (Mires, 1983; Liao and Chen, 1983 ; Kissil, 1996). However, this cross gives inconsistent male percentages of 59-81% which is thought to be caused by introgression of hybrid fry back into the pure parental gene pools. Wohlfarth (1994) recommended the necessity of purity testing of parental stocks and establishing a pilot scheme for hybrid tilapia production on a semi-commercial scale as the first step in adoption of the approach. More recently, Marengoni *et al.* (1998) attempted to produce all male progeny in crosses between *O. niloticus* (Local-Egypt and Stirling strains) X *O. aureus* and the proportion of males were 91% for local-Egypt and 100% for Stirling strain. On the other hand, Hulata *et al.* (1993) reported 30% male progeny in crosses of *O. niloticus* (Ghana-88 strain) X *O. aureus* (Mehadrin strain) crosses. Mair (1988) suggested that different geographical strains of the same species possess genetic variation for various genes involved in sex determination, which could contribute significantly to the differences in sex ratios.

Pruginin *et al.* (1975) summarised the interspecific crosses in tilapia that produce skewed sex ratios. The crosses between *O. niloticus* X *O. variabilis*; *O. nigra* X *O. hornorum*; *O. vulcani* X *O. hornorum* and *O. vulcani* X *O. aureus* produced 97-100% male progeny. Majumdar and McAndrew (1983a) produced 100% male hybrids in only one cross of *O. mossambicus* X *O. macrochir* and 97.9% male hybrid in *O. spilurus* X *O. macrochir*.

The variable sex ratios in progeny of different crosses is not predicted from the crosses of fish having: a simple monofactorial system of a homogametic female (XX) and a heterogametic male (XY) as in *O. niloticus* and a heterogametic female (WZ) and a homogametic male (ZZ) as in *O. aureus* (Trombka and Avtalion, 1993). In the cross of *O. niloticus* X *O. aureus*, 100% male progeny can be produced while the reciprocal cross of *O. aureus* X *O. niloticus* would be expected to produce 3 male and 1 female sex ratio in offspring. The possible explanation for aberrant sex ratios produced from interspecific crosses will be given in section 1.4.

Although hybridisation offers several advantages such as avoiding inbreeding and use of sex hormones and combining of desirable characteristics from two species (e.g. faster growth rate of *O. niloticus* and cold tolerance of *O. aureus*), there are a number of problems associated with the mass production of all male hybrids. Firstly, the success or failure of tilapia hybridisation in producing all male hybrid progeny is initially dependent on the choice of species to be hybridised. Secondly, genetic purity of parental stocks is essential for successful hybridisation. This is especially difficult in tilapia because of the similarity of hybrids and their parental species. There is also an apparent inherent instability of all male hybrid production. The proportion of males decreased when mass production for commercial culture was attempted (Hulata *et al.*, 1983, 1985; McAndrew, 1993; Wohlfarth, 1994). Thirdly, low hybrid fry production due to incompatibility between species is another constraint (Lovshin, 1982; Mires 1982; Hulata *et al.*, 1985).

There appears to be little or no heterosis associated with interspecific hybridisation in tilapia, therefore the optimum performance of a species in a specific environment can be lost by crossing it with the other species with poor performance. For

example in crosses of *O. niloticus* X *O. aureus*, although all male and cold tolerant fish population could be produced, growth potential of *O. niloticus* could be lost. A major problem of this technology is the loss of pure strains through intentional and unintentional hybridisation and therefore its widespread use would constitute a major loss of biodiversity (Mair and Little, 1991).

1.4.4. Hormonal sex reversal to male

Because of the difficulties in obtaining pure species for hybridisation, more consistent methods were needed to produce all male fish. The first sex reversal achieved in a fish, the medaka, *Oryzias latipes* in both directions i.e. from male to female and from female to male was done by incorporating sex steroids into food (Yamamoto, 1969). After that, this technique has been widely developed up to commercial-scale applications in many species where one of the two sexes has superior culture characteristics.

Sex reversal from genetic female to phenotypic male using male androgens has been successfully carried out in medaka, goldfish (*Carassius auratus*), tilapia species, zebra fish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tschawytscha*) (Yamazaki, 1983).

The efficacy of an androgen (male hormone) is affected by the mode of administration and by its source, whether synthetic or naturally occurring (White *et al.*, 1973). Among the total of 16 androgens tested (5 natural and 11 synthetic) synthetic androgens such as ethynyltestosterone (ethisterone) and methyltestosterone are more effective when administered orally than naturally occurring androgens like testosterone,

androsterone, andrenosterone and androstenedione which are more potent when injected intraperitoneally which is not possible for early life stages of fish (Tayamen and Shelton, 1978; Pandian and Sheela, 1995). 17α-methyltestosterone has proved to be both effective and relatively inexpensive for masculinising tilapia fry and has been preferred by most workers (Mair and Little, 1991; Macintosh and Little, 1995; Pandian and Sheela, 1995).

Yamamoto (1969) established criteria for effective hormonal sex induction of fish, which have become the principles for hormonal sex manipulation in many fish species. These are,

1) that steroids should be administrated to sexually undifferentiated fish and treatment should be continued until the end of sex differentiation and
2) that the species-specific optimal dosage of a particular steroids should be used.

A very high percentage of male fish can be obtained by the administration of androgens to sexually undifferentiated tilapia fry. The onset of sexual differentiation in tilapia depends on the species and environmental factors, but generally it occurs between 16-20 days after hatching; thus hormone treatment must cover this sensitive period for sex reversal to be effective (Shelton *et al.*, 1978).

Production of all male or nearly all male tilapia by the oral administration of androgens to sexually undifferentiated fry has been achieved by several workers (Clemens and Inslee, 1968; Guerrero, 1975, 1982; Tayamen and Shelton, 1978; Hopkins, 1979; Shelton *et al.*, 1981; Mair *et al.*, 1987b; Mair and Little, 1991; Vera Cruz and Mair, 1994).

The choice of dosage is critical to treatment efficiency. Several interrelated factors influence the optimal dosage level of a particular steroid including its biological activity

(which may be related to its origin), the route of administration, the target species and the duration of treatment (Hunter and Donaldson, 1983). In *Oreochromis*, a dosage of 17α-methlytestosterone ranging from 30-60 mg/kg diet was reported as an effective level for sex reversal to male (Guerrero, 1975; Tayamen and Shelton, 1978; Calhoun and Shelton, 1983; Macintosh *et al.*, 1988; Macintosh and Little, 1995; Argue and Phelps, 1996). On the other hand the optimum dose required to induce sex reversal appears to be species-specific. Yamazaki (1983) reported that 30 mg/kg diet is effective for *O. mossambicus* and *O. niloticus* while 30-60 mg/kg diet is appropriate for *O. aureus*. Hunter and Donaldson (1983) and Yamazaki (1983) concluded that the sensitive period to hormonal induction for sex reversal varies not only from species to species but also from family to family. Clemens and Inslee (1968) and McAndrew and Majumdar (1989) produced 100% male using methyltestosterone at 10-40 mg/kg diet for 60 days and 40 mg/kg diet for 40 days in *O. mossambicus*, respectively. All male production was achieved from *O. niloticus* fry exposed to 17α-methyltestosterone at a dose of 40, 30-60 and 50-100 mg/kg for a duration of 60, 25-29 and 30 days by Jalabert *et al.*, (1974), Tayamen and Shelton (1978) and Nakamura and Iwahashi (1982), respectively. Shelton *et al.* (1981) concluded that the duration of hormonal treatment is more critical to success than factors that affect growth. The authors reported that high stocking density of 2600 fry m^{-2} reduced the growth rate but did not reduce the success of sex reversal in *O. aureus*. Varadaraj *et al.* (1994) also observed that stocking density did not affect the sex ratio but significantly reduced the growth and survival of 17α-methyltestosterone-treated *O. mossambicus* fry.

Successful sex reversal can also be affected by environmental factors e.g. temperature and photoperiod (Shelton *et al.*, 1981; Varadaraj *et al.*, 1994). Mair and Little (1991)

summarised the related conditions associated with effectiveness of hormonal sex reversal production in tilapia species (Fig. 1.2.)

Hormonal sex inversion is routinely applied in many countries to first-feeding tilapia fry to produce all male populations for restocking into production ponds to overcome the problem associated with mixed sex culture (McAndrew and Majumdar, 1989). However, there are a number of disadvantages to hormonal sex reversal that constrain its application world-wide. These are:

1) the availability and the cost of hormone with high quality feed ingredients,
2) varying success with sex reversal using standard techniques,
3) mass production of sexually undifferentiated fry suitable for sex reversal treatment,
4) consumer reaction to hormone treated fish,
5) potential health risk of hormone to pond workers,
6) ecological impact of using hormone,
7) growing awareness of environmental impact,
8) legislative aspects.

The development of hybrid and sex reversed fish enabled experimentation on the nature of the sex determination mechanism in tilapia which eventually enable genetic control of sex *in O. niloticus*. The development of our understanding of the sex determining mechanism in tilapia will now be discussed.

1.5. Sex determination in tilapia

Understanding the sex determining mechanism in fish is of very great importance for the production of monosex fish populations. There have been four main approaches considered to investigate the mechanism of sex determination in tilapia: 1) inter and intra-

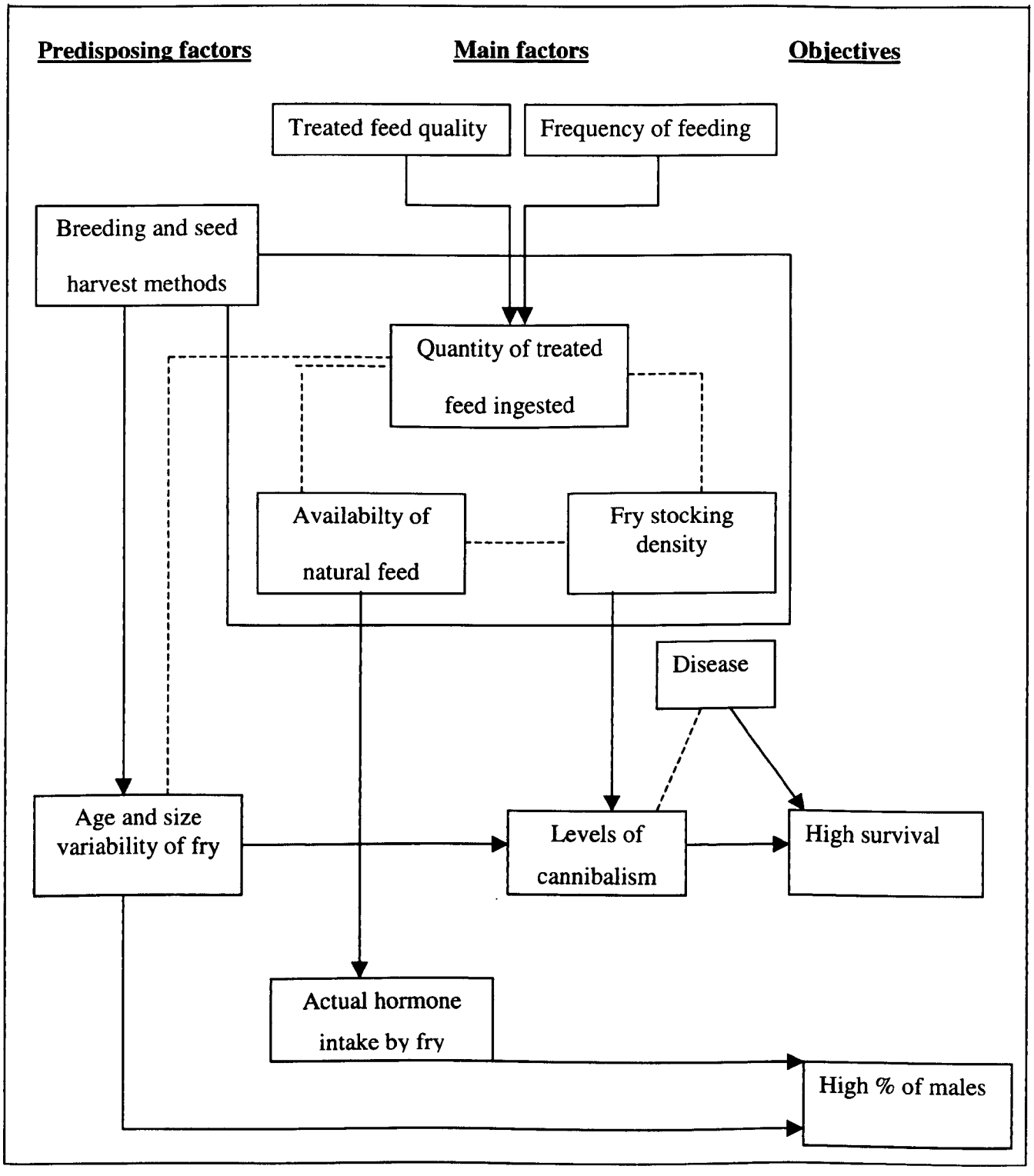


Figure 1. 2. Factors affecting the efficacy of hormonal sex reversal of tilapia. (Source: Mair and Little, 1991). Legends: —— Major relationship, ------ Minor relationship

species crosses (Pruginin *et al.*, 1975; Hulata *et al.*, 1983; Shelton *et al.*, 1983; Majumdar and McAndrew, 1983a; Mair, 1988; Tuan, 1997; Mair *et al.*, 1991a,b); 2) sex reversal (Hopkins *et al.*, 1979; Mair *et al.*, 1987b; Avtalion and Don, 1990; Mair *et al.*, 1991a,b; Baroiller, 1996); 3) chromosomal manipulation (Penman *et al.*, 1987; Shah, 1988; Mair *et al.*, 1987a, 1991a,b; Peruzzi *et al.*, 1993, Muller-Belecke and Horstgen-Schwark, 1995) and 4) analysis of karyotypes and sex-linked markers (Avtalion *et al.*, 1976; Avtalion, 1982; Majumdar and McAndrew, 1983b; Nijhar *et al.*, 1983; Carrasco *et al.*, 1999).

1.5.1. Inter and intra-specific crosses

The interspecific crosses between some species of tilapia result in all male or predominantly male offspring (Hickling, 1960; Chen, 1969; Pruginin *et al.*, 1975; Hulata *et al.*, 1983; Majumdar and McAndrew, 1983a; Lahav and Lahav, 1990; Wohlfarth *et al.*, 1990). Although these interspecific crosses have been aimed to produce all male progenies commercially, they also offer a means to understanding the sex determining mechanism in tilapia. The results from interspecific crosses between *Oreochromis* species suggest a dual sex determining system in this genus which is homogametic female (XX) and heterogametic male (XY) e.g. *O. niloticus* and *O. mossambicus* and heterogametic female (WZ) and homogametic male (ZZ) e.g. *O. hornorum* and *O. aureus*. Chen (1969) suggested that all male hybrid broods could be obtained when homogametic males of one species are crossed with homogametic females of another species. However, the sex ratios in hybrid crosses are variable and the theory of Chen (1969) cannot explain satisfactorily all the sex ratios observed by several authors (Pruginin *et al.*, 1975;

Wohlfarth and Hulata, 1981; Majumdar and McAndrew, 1983a; Hulata *et al.*, 1983; Mair *et al.*, 1991b).

Several explanations for the deviation of sex ratios from the expected were hypothesised such as contamination of parental stock by another species, autosomal influence including polygenic sex determination, differential mortality of certain genotypes and environmental factors (Yamamoto and Kajishima, 1968: cited in Trombka and Avtalion, 1993; Avtalion and Hammerman, 1978; Shelton *et al.*, 1983; Mair *et al.*, 1990; Wohlfarth and Wedekind, 1991).

The possible involvement of autosomal and gonosomal influences was suggested by Avtalion and Hammerman (1978) and Hammerman and Avtalion (1979). According to their theory, the system of sex determination in tilapia has three gonosomes (X, W and Y) in any one of the possible combinations (XX, XY, WX, WW, WY and YY) and a pair of autosomes (AA, Aa and aa) which are involved in primary sex determination. Under this theory, within each pure species the pairs of autosomes are identical and 18 different genotypes are predicted (Table 1.4). Thus, the set of chromosomes present in the pure species of some commercially important species would be

	Female	Male
Genotype I	AAXX	AAXY (*O. mossambicus*, *O. niloticus*)
Genotype II	aaWY	aaYY (*O. hornorum*, *O. macrochir*, *O. aureus*)

Although this hypothesis was able to explain most of the sex ratios obtained by Chen (1969), it still fails to explain the large variation observed in hybrid sex ratios in repeated crosses (Pruginin *et al.*, 1975; Hulata *et al.*, 1983; Majumdar and McAndrew, 1983a;

Table. 1. 4. Influence of autosomal pairs on sex determination in interspecific crosses in tilapia species (Source: Avtalion and Hammerman, 1978). m: male, f: female

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

Mair *et al.*, 1991b) and also presence of males among gynogenetic tilapia (Hussain *et al.*, 1994; Mair *et al.*, 1991a,b; Muller-Belecke and Horstgen-Schwark, 1995).

Majumdar and McAndrew (1983a) suggested a polygenic system in tilapia based on their hybrid sex ratio results using pure species analysed by protein electrophoresis, which negate the hypothesis of xenogenic infiltration (introgression of gene pool from different species).

In recent years most researchers have concentrated on intraspecific sex ratios to investigate sex determining mechanisms in tilapia (Shelton *et al.*, 1983; Majumdar and McAndrew, 1983a; Tuan, 1997; Mair, 1988; Lester *et al.*, 1989; Mair *et al.*, 1991a; Wohlfarth and Wedekind, 1991; Marengoni *et al.*, 1998). Shelton *et al.* (1983) reported

that the sex ratios of *O. niloticus* from about 5500 offspring ranged from 31-83% males with a mean of 54.7%, showing a normal distribution while the sex ratios of *O. aureus* from about 9000 offspring ranged from 0-100% males with a mean of 49.4% in which sex ratios of 20% of the 126 progeny groups were statistically different from the expected 1:1 (male: female) ratio. The authors concluded that sex determination in tilapia is more complicated than single monofactorial heterogamety. In a similar study involving 59 single-pair matings of *O. niloticus*, sex ratio varied between 2-62% male with a slight excess of females (Lester *et al.*, 1989) The hypothesis of multifactorial mechanisms in sex determination in tilapia were suggested by Shelton *et al.* (1983), Majumdar and McAndrew (1983a) and Lester *et al.* (1989).

Scott (1988) and Mair *et al.* (1991a) studied the mechanism of sex determination in the Egypt-Swansea (Stirling) strain of *O. niloticus* from 22 and 59 progeny groups, respectively. The overall sex ratios differed significantly from the 1:1 expected ratio with a slight excess of males (55.1% and 53.12%, respectively). The first author explained the aberrant sex ratios by the existence of naturally sex reversed females (XY).

Mair (1988) attempted to investigate the sex determining mechanism in Egypt-Swansea strain of *O. niloticus* by complete diallele-type crosses using 5 females and 5 males. He reported that there was a lack of paternal and maternal effect on sex ratios in this strain which shows female homogamety and male heterogamety.

The sex ratios in 22 intraspecific progeny groups from single-pair mating of local-Egypt strain and Stirling strain of *O. niloticus* gave a mean male percentage of 50.8% and 48.6%, respectively, which was not significantly different from the expected sex ratio of 1:1 while the sex ratios of *O. aureus* from 22 single pair matings were significantly

different from the expected sex ratio of 1:1 (Marengoni *et al.*, 1998). The authors explained the disparity in sex ratio in *O. aureus* by effects of autosomal modifier alleles.

1.5.2. Sex reversal

Sex reversal has been used by several tilapia researchers to elucidate the sex determining mechanism in tilapia (Clemens and Inslee, 1968; Guerrero, 1975, 1979; Hopkins *et al.*, 1979; Mair *et al.*, 1987b, 1991a,b; Baroiller, 1996; Lahav, 1993). The basic approach is to analyse sex ratios of offspring from treated parent fish crossed to either normal males or females. Clemens and Inslee (1968) obtained all female progeny by crossing neomales (XX) to normal females, suggesting female homogamety in *O. mossambicus*. In similar work, Mair *et al.* (1991a) crossed neomales *O. niloticus* to normal females, which resulted in all females. The authors suggested female homogamety in this species.

Sex ratios of progeny from hormonally sex reversed fish were also analysed in *O. aureus* (Guerrero, 1975; Hopkins *et al.*, 1979; Mair *et al.*, 1987b, 1991b; Lahav, 1993; Desprez *et al.*, 1995). Hopkins *et al.* (1979) reported slight deviations from the expected 100% male progeny derived from the crosses of neofemales (ZZ) and normal males (ZZ). The same observation was also reported by Mair *et al.* (1991b) who produced large proportions of females (18-40.2%) from crosses of neofemales to normal males of *O. aureus*. These authors claimed the presence of modifying factors altering sex ratios from those predicted by a monofactorial model.

1.5.3. Environmental sex determination

Deviation from the expected sex ratio in progeny derived from sex reversed *O. niloticus* was also claimed to be due to environmental factors in sex determination (Mair *et al.* 1990; 1991a,b). This suggestion was further supported by Baroiller (1996) based on sex ratio of progenies from single pair matings of normal females and neomales of *O. niloticus*.

There are several examples of environmental sex determination (ESD) in the animal kingdom (Bull, 1983). Although most of the work has involved turtles and other reptile species (Bull *et al.*, 1982; Dournon *et al.*, 1990), ESD has been reported in several fish species such as Atlantic silverside, *Menidia menidia* (Conover and Kynard, 1981), the livebearing teleost fish, *Poeciliopsis lucida* (Sullivan and Schultz, 1986), atherinid fishes, *Odontesthes bonariensis* and *Patagonina hatcheri* (Strüssman *et al.*, 1997) and loach, *Misgurnus anguillicaudatus* (Arai *et al.*, 1997).

Temperature effects on sex ratios of tilapia species have been reported by several authors (Mair *et al.*, 1990; Baroiller *et al.*, 1995a,b; Baroiller, 1996; Desprez and Mélard, 1998; Abucay *et al.*, 1999) and more detail will be given in Section 5.1.2.2.

1.5.4. Gynogenesis and androgenesis

Induced gynogenesis and androgenesis are processes of reproduction in which normal eggs are fertilised with UV inactivated sperm and UV or gamma inactivated eggs are fertilised with normal sperm, respectively. As a result, the embryonic development takes place with the inheritance of only the maternal (gynogenesis) or paternal (androgenesis) chromosome set. Diploidization of haploid zygotes can be made by suppressing the

second meiotic division in case of gynogenesis ("meiotic" gynogenetic fish or "meiogynes") or first mitotic division in the case of gynogenesis and androgenesis ("mitotic" gynogenetic fish or "mitogynes" and "androgenetic" fish, respectively) by means of physical shocks such as temperature or pressure. In this respect, both gynogenesis and androgenesis are valuable techniques to elucidate sex determination mechanisms in fish. Alhough gynogenesis has been used for analysing the sex determination mechanisms in tilapia (Penman *et al.*, 1987; Shah, 1988; Mair *et al.*, 1987a, 1991a,b; Hussain *et al.*, 1994; Sarder *et al.*, in press), low survival of androgenetic tilapia has constrained the use of this technique for analysing the sex determination mechanism in tilapia species.

Penman *et al.* (1987) and Shah (1988) obtained only female meiotic gynogenetic progeny in *O. niloticus*, suggesting monofactorial sex determination system with female homogamety and male heterogamety. Howevèr, Mair *et al.* (1991a) reported 7.5% and 47.5% males while Hussain *et al.* (1994) observed 4.1% and 20% males in meiotic and mitotic gynogenetics, respectively in *O. niloticus*. 35.3% mitotic males were also reported by Muller-Belecke and Horstgen-Schwark (1995) in *O. niloticus*. Hussain *et al.* (1994) proposed an epistatic locus (*SDL-2*, two alleles, SR and sr) causing sex reversal of female to male under homozygous condition. Mair *et al.* (1991a) explained their unexpected males as being due to the "homozygosity of rare autosomal, recessive, sex influencing genes" causing some form of natural sex reversal of females.

Penman *et al.* (1987) and Mair *et al.* (1991b) induced gynogenesis in *O. aureus*. The sex ratios of gynogenetic *O. aureus* were significantly different from the expected 1:1 ratio with a large excess of females. These results were explained by hypotheses of

recombination of sex determining genes during the prophase of the first meiotic division. Therefore, a single crossover would produce all female (WZ) progeny and double crossover would yield equal number of males and females while recombinant females would occur depending on the rate of crossing over between the sex determining gene and centromere.

1.5.5. Karyotyping and sex linked markers

Sex linked phenotypes such as colour or biochemical markers have not been demonstrated in tilapia (Trombka and Avtalion, 1993). Avtalion *et al.* (1976) identified a male sex–specific protein (MSP) in tilapia. However, this protein is found in small amounts in female as well and cannot be considered as a sex-linked marker.

Studies failed to show heteromorphic sex chromosomes in the majority of fish species (Yamazaki, 1983). A simple chromosomal classification based on size, centromere position and differential staining did not show any obvious evidence for sex chromosomes in tilapia (Kornfield, 1984; Majumdar and McAndrew, 1983b). Nijhar *et al.* (1983) reported heterogamety in *O. niloticus* based on the differences in the length of the two long chromosomes. However, the evidence presented is equivocal since the precise stage of mitosis and the degree of DNA condensation can change the morphological and banding properties of the samples (Buys *et al.*, 1983).

The study of the synaptonemal complex in paired chromosomes in the late zygotene and pachtene in homogametic and heterogametic males of *O. niloticus* showed an incompletely paired segment in most samples in the terminal region of the largest

bivalent of XY males providing cytological evidence for the chromosomal basis of sex determination in this species (Foresti *et al.*, 1993; Carrasco *et al.*, 1999).

1.6. YY male production

Development of a technique for commercial production of all male tilapia population by production of YY male broodstock is another approach since the direct use of hormones on the final product could be avoided. Based on a number of studies on the sex determining mechanism in *O. niloticus*, a predominantly monofactorial mechanism with female homogamety (XX) and male heterogamety was proposed (Jalabert *et al.*, 1974; Penman *et al.*, 1987; Mair *et al.*, 1987b, 1991a; Mair, 1988; Tuan, 1997). Based on that assumption, a model was proposed for production of genetically all male progeny through generating of YY male genotypes by incorporating endocrine sex reversal and selective breeding technique. A schematic diagram depicting the model for large-scale production of YY males is presented in Fig 1.3.

The first stage of programme involves generation of neofemales (XY) using fry produced from normal crosses of females (XX) and males (XY) by using a synthetic estrogen, diethylstilbestrol (DES). XY neofemales can be identified by the progeny testing with either normal males (XY) or neomales (XX) and the sex ratio of 3:1 or 1:1 (male: female), respectively, would be expected. YY males can easily be distinguished by progeny testing with normal females (XX) resulting in all male progeny. The main problem is that of generating large numbers of YY males for use as broodstock in a commercial system. Mass production of YY males can be achieved by feminisation of

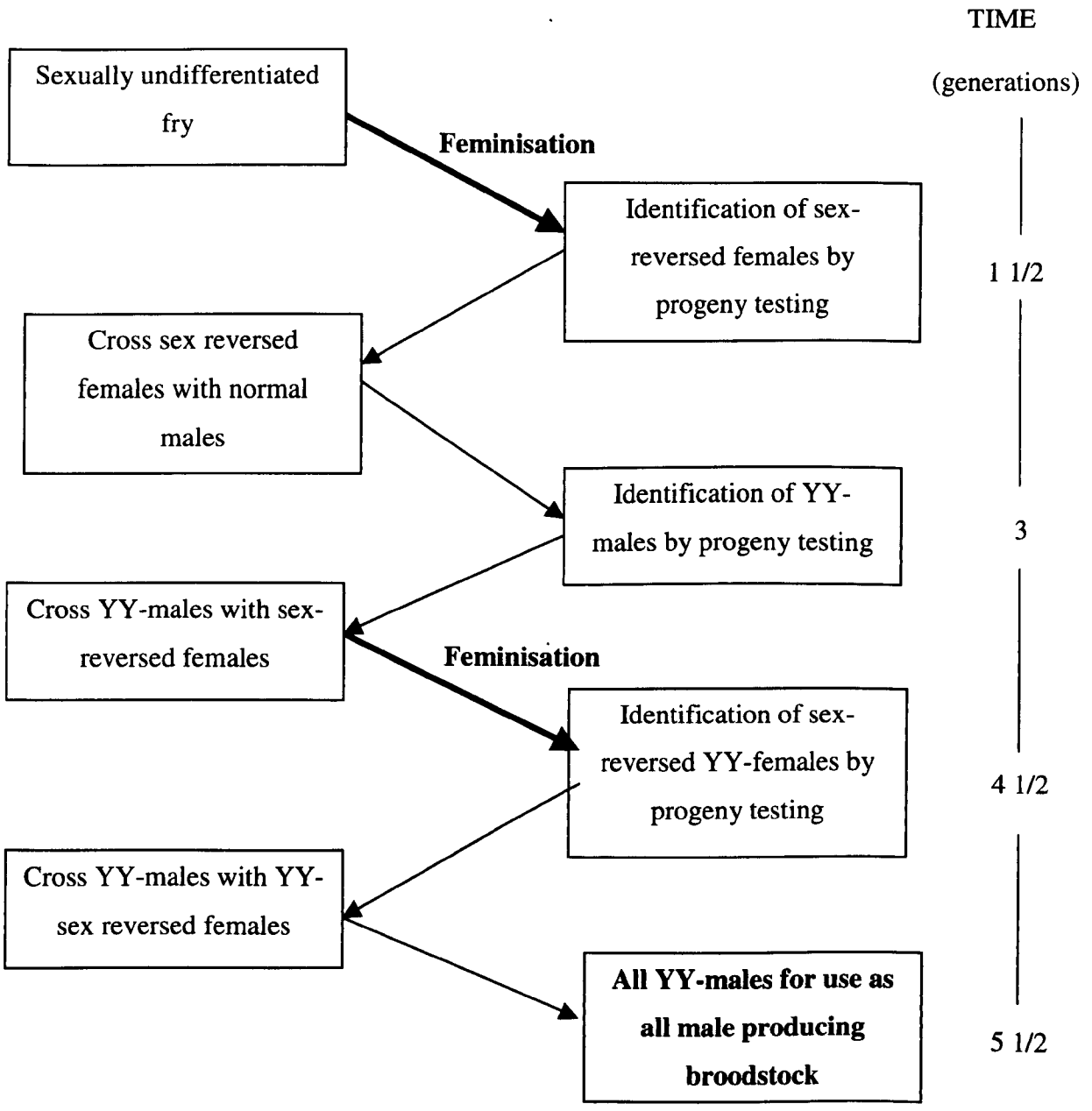


Figure 1. 3. Schematic diagram depicting the model for large scale production of YY males (Source: Mair *et al.*, 1993).

progeny of YY males crossed to neofemales (XY) to produce YY neofemales which then be crossed to YY males.

The YY males have been to shown to be as viable and fertile as normal males and produce sex ratios ranging from 71.6-100% male with a mean of 97.3% male when crossed to normal females (Mair *et al.*, 1993). Mair *et al.* (1995) reported that genetically all male tilapia derived from YY male had faster growth rate and greater survival compared to androgen treated phenotypically all male and mixed sex populations. The YY male production has only been applied to the Egypt-Swansea (Mair *et al.*, 1997) and Thai-Chitralada (Tuan *et al.*, 1999) strains of *O. niloticus* and further research is necessary to evaluate and improve this technique for other strains of *O. niloticus.*

1.7. Red tilapia

The term "red tilapia" is used to describe a strain or an individual showing a range of various colours either singly or in combination from white through pink, red, orange and gold, with or without black blotching. McAndrew and Wohlfarth (in press) review the literature and the main red tilapia strains used commercially worldwide. The major cultivated red strains are mostly hybrids involving as many as four species in which mainly red *O. mossambicus* has been hybridised with other faster growing species or their various strains and isolates (*O. niloticus*, *O. aureus* and *O. hornorum*) and selected for colour to improve their culture performance (McAndrew and Wohlfarth, in press).

Taiwanese red tilapia (TRT) is thought to be a hybrid between red *O. mossambicus* X *O. niloticus* (Liao and Chen, 1983). However, isolates of the TRT have also been further hybridised with *O. niloticus* and *O. aureus* in Israel (Wohlfarth *et al.*,

1990) and with *O. niloticus* in Puerto Rico (Verdegrem, 1987: cited in McAndrew and Wohlfarth, in press).

Philippine red tilapia (PRT) was said to originate from the crosses between *O. mossambicus* and Philippine strain of *O. niloticus*. Galman *et al.* (1988) describes at least six different phenotype in this strain; red/gold/orange with black spots, uniform pink, pink with black spots, albino with black eyes and grey.

The Florida red tilapia (FRT) originally developed by Sipe (1979) from an original mutant white *O. mossambicus*. The white *O. mossambicus* was extensively used in hybrid crosses with *O. niloticus*, *O. aureus* and *O. urolepis hornorum*.

Since Stirling red *O. niloticus* was used in the present study, more detail will be given on this strain. The origin of Stirling red strain of *O. niloticus* is described by McAndrew *et al.* (1988). According to the authors, a single red male tilapia was observed in a batch of wild type fry of a pure strain of *O. niloticus* originating from Lake Manzala, Egypt being maintained in the Tilapia Reference Collection at the Institute of Aquaculture, University of Stirling. The same authors worked on the red, blond and other associated colour variants in this strain. In this study, the structure of skin and distribution of chromatophores was analysed by using both light and electron microscope. The pigmentation of the skin of the wild type fish shows normal pigmentation in that there is a delicate layer of melanophores (black pigments) with some iridophores (silver pigments) immediately below the epidermis, in the stratum spongiosum, and a denser layer comprising iridophores and melanophores in the hypodermal tissue. Erythrophores (red pigments) and xanthophores (yellow pigments) are lightly distributed in both layers. Red fish and red areas of black blotched fish contained no melanophores. On the other

hand, black areas on the red fish showed normal melanophores. Blond fish are characterised by the lack of pigmentation in the fertilised eggs and a pale appearance showing an unpigmented body cavity. There are no pigments in the stratum spongiosum, the hypodermis having only occasional reduced black pigment cells and a slightly thickened layer of iridophores.

There appear to be a number of different genetic mechanisms controlling skin colour in the different strains and sometimes even in the same strain of red tilapia. A number of papers are available about the colour inheritance mechanism in red tilapia which were recently reviewed by McAndrew and Wohlfarth (in press).

McAndrew *et al.* (1988) reported that the red mutation is an autosomal dominant with two alleles (RR) over the wild type (rr) in Stirling red *O. niloticus*. Crosses between red (RR) and wild type (rr) produced heterozygotes individuals exhibiting a range of black blotching from 0-24.6% of the skin surface. On the other hand, homozygous red fish were reported to be always free of any blotching but sometimes had a few isolated black spots especially around eyes. They claimed that selection for blotch free fish produced a strain which is free of black pigmentation even in heterozygous fish. They also hypothesised that the blotched phenotype is controlled by a single gene with two alleles, the dominant being responsible for blotching and the recessive for a lack of blotching. Hussain *et al.* (1994) and Koren *et al.* (1994) working on same Egyptian red tilapia strain also supported the hypotheses that a single autosomal dominant "R" gene controls red body colour. Plate 1.1. and 1.2. show the different colour types used in the present study.

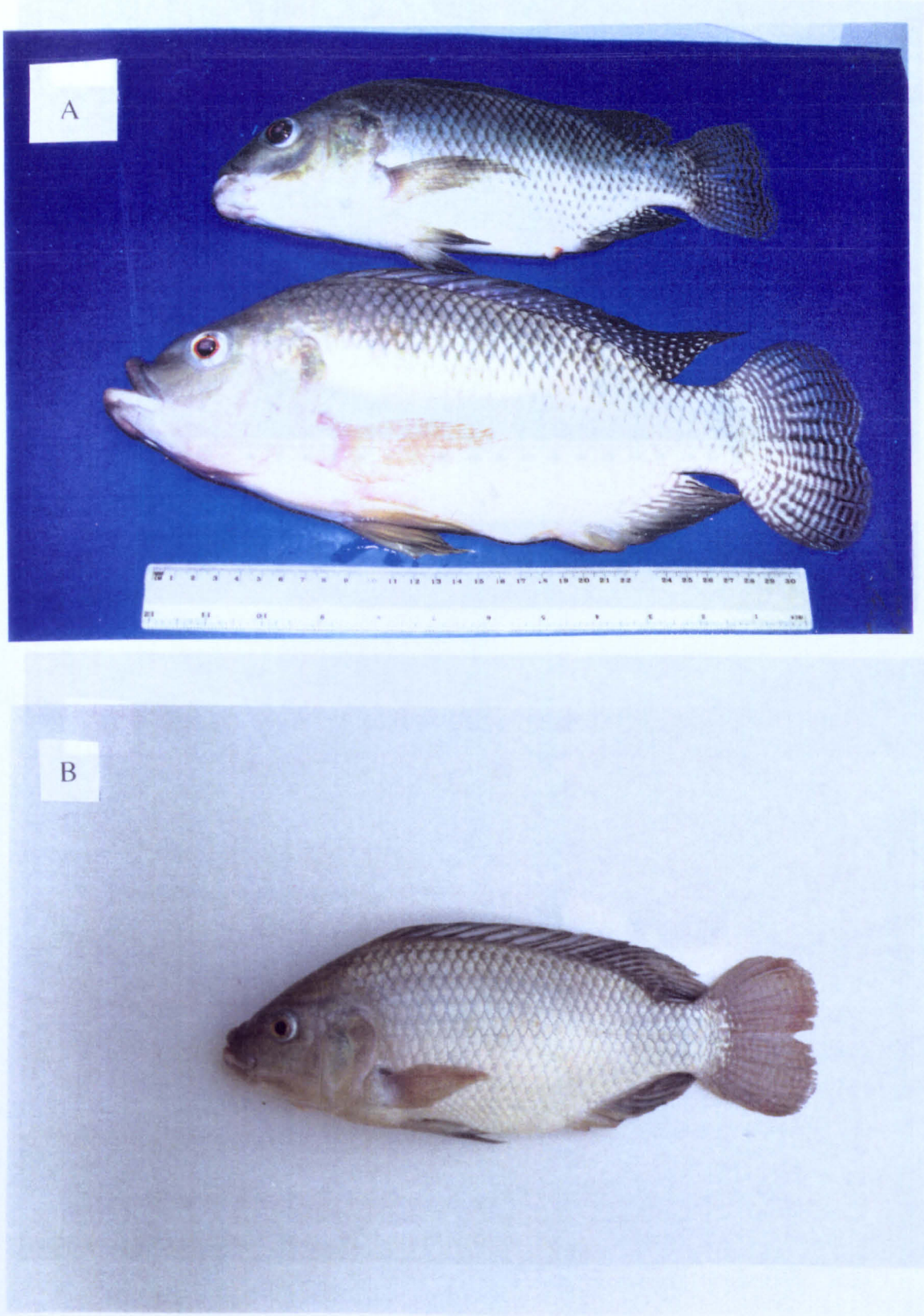
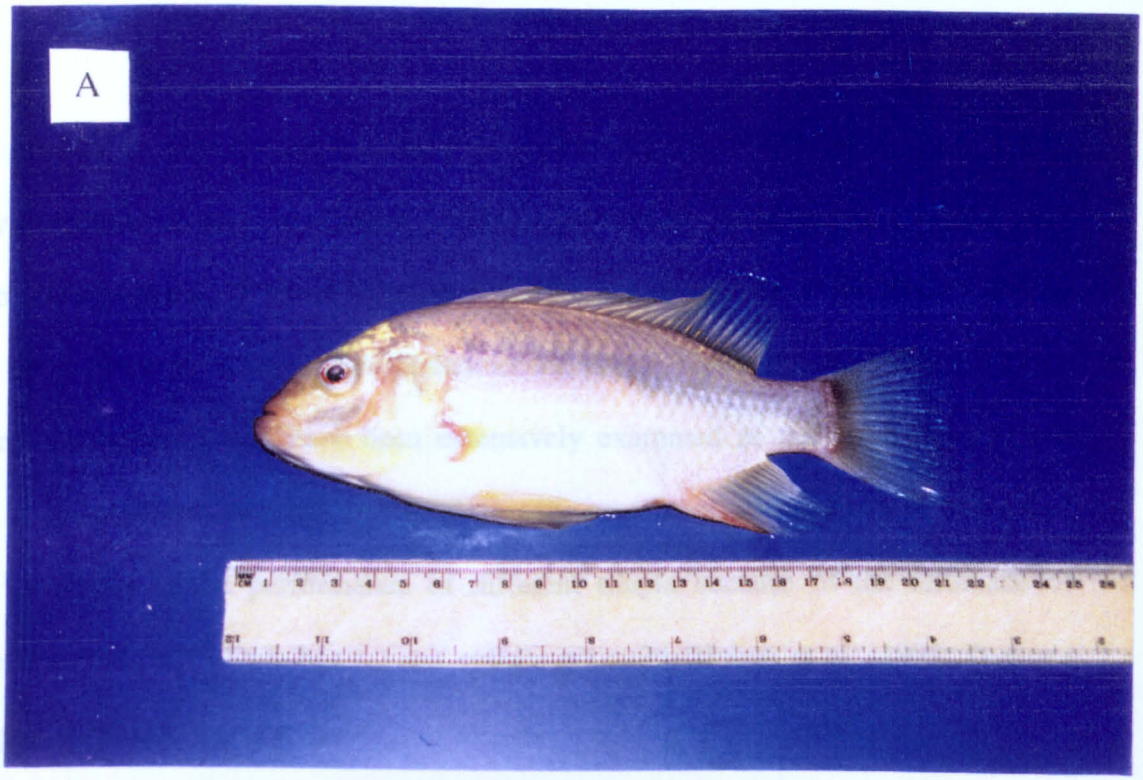


Plate 1.1. (A) Normal wild type (upper a female, lower a male) and (B) blond *O. niloticus*.



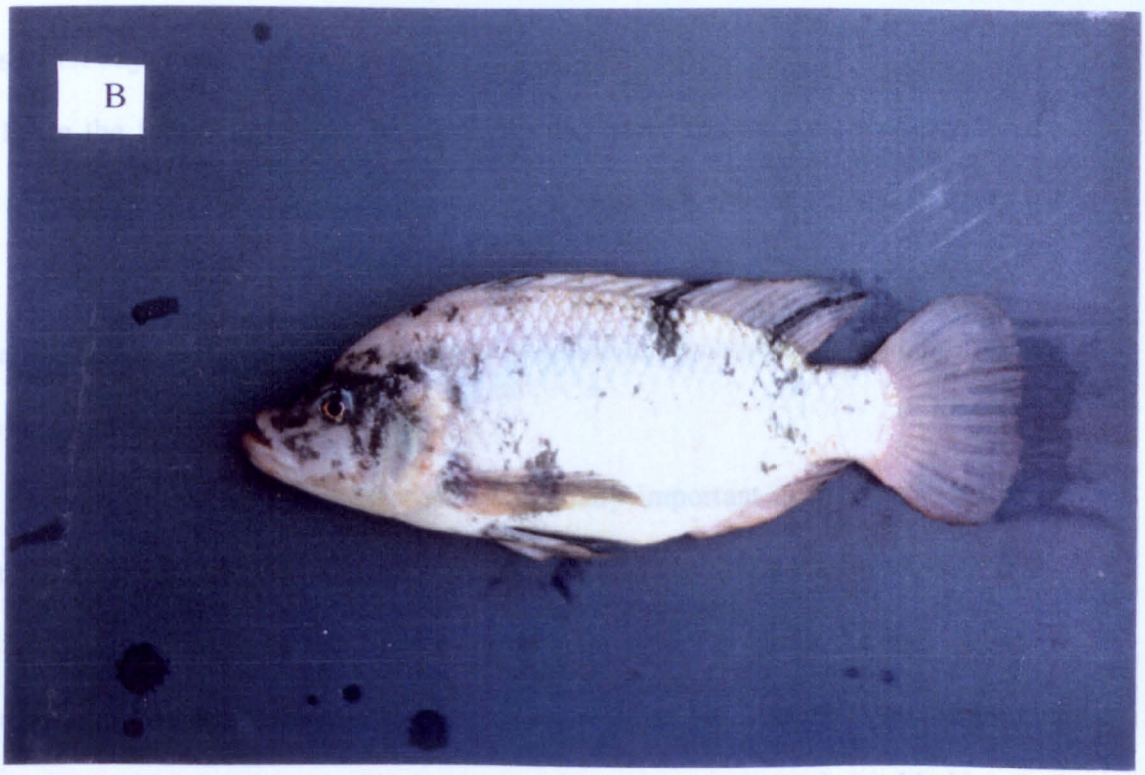


Plate 1.2. (A) Red and (B) blotched *O. niloticus*.

Production related traits such as cold tolerance (Behrends and Smitherman, 1984), salinity tolerance (Liao and Chang, 1983), reproductive performance (Galman and Avtalion, 1983; Eguia, 1996) and growth performance, (Behrends *et al.*, 1982; Pruginin *et al.*, 1988; Galman *et al.*, 1988; Hulata *et al.*, 1995; Siddiqui and Al-Harbi, 1995; Deguara and Aguis, 1997) have been extensively examined as the importance of red tilapia in global aquaculture has increased over the past years. Although there was found to be variability in the performance of different tilapia because of the influence of particular strains, red tilapia has a great culture potential (Hulata *et al.*, 1995; Deguara and Agius, 1997).

1.8. Research objectives

From the foregoing, it can be seen that two main factors are necessary for the genetic improvement of red tilapia: good stable colour which were not found in many red strains and the ability to produce all male fry. In the red *O. niloticus* strain held at Institute of Aquaculture, Stirling, it should be possible to combine these traits in a single strain using genetic techniques.

The genetic improvement of any commercially important phenotype in terms of production has been attempted for years in agriculture and by livestock breeders. It relies mainly on the knowledge of the genetic mechanism which controls the expression of these useful phenotypes. Red colouration in tilapia has become an important characteristic and utilisation in tilapia improvement has increased worldwide. Red colouration and genetically all male populations are currently available in separate strains of *O. niloticus*. Therefore, the main objectives of the studies in this thesis were to

investigate options for the combination of both valuable traits in a single pure *O. niloticus* strain. However, the knowledge of the mechanism of sex determination and colouration is fundamental to the success of production of all red and male *O. niloticus*. Therefore, the following approaches were taken in these investigations:

1) Improvement of androgenesis in the Stirling red strain of *O. niloticus* for production of YYRR males and YYRR neofemales in only one generation.
2) Generation of YYRR males and YYRR neofemales by introgression of YYrr males and YYrr neofemales of the Egypt-Swansea isolate and Stirling red isolate of Egyptian strain *O. niloticus*.
3) Generation of pure bred YYRR males and YYRR neofemales using existing androgenetic YYRR males in Stirling red isolate of Egyptian strain of *O. niloticus*.
4) Further investigations into mechanisms of sex determination and colouration in *O. niloticus* through androgenesis, gynogenesis and studying sex and colour ratios in progenies during the development of YYRR males and YYRR neofemales.
5) Investigation of the effect of the colouration on reproductive traits and survival rate in Stirling red and wild type strain of *O. niloticus*.

Chapter 2:

Production of androgenetic Nile tilapia, *O. niloticus*

2. Production of androgenetic red Nile tilapia, *O. niloticus*

2.1. Introduction

The external fertilisation of gametes in fish provides a possibility to manipulate their chromosome numbers. Chromosome-set manipulation techniques in fish have been subjected to extensive study because of their potential for use in sex control, rapid production of inbred lines and genetic analyses of important traits. Techniques are available which can produce haploid, or polyploid fish and even produce fish whose chromosomes come solely from either their mothers (gynogenesis) or from their fathers (androgenesis).

2.1.1. Gynogenesis

Gynogenesis involves fertilising eggs with inactivated sperm and prevents any contribution of the male genome to the embryo. It was first described by Hertwig (1911: cited in Thorgaard, 1983) in frog embryos, after spermatozoa had been irradiated with radium gamma rays, prior to fertilisation. Hertwig observed that low doses do not totally destroy the sperm genome, yet this results in lower apparent larval survival than high doses which induce complete inactivation of the genome resulting in an increase in haploid embryos and an apparent improvement in larval survival. The chromosome fragments in the low dose treatments are thought to be lethal to larva. This dose response became known as the Hertwig effect. Two years after Hertwig's experiments, Opperman

(1913: cited in Thorgaard, 1983) became the first author to test the effect of increasing doses of ionizing rays from radium and thorium on the sperm of brown trout, *Salmo trutta*. He observed a typical "Hertwig effect". Since the work of Hertwig, many authors have used ionizing rays, ultraviolet rays and chemical mutagens to produce haploid gynogenetic fish (see reviews by Thorgaard, 1983; Chourrout, 1987; Ihssen *et al.*, 1990; 1996).

Ionizing radiation (X rays and ^{60}Co gamma rays) has a good penetrating power that facilitates treatment of large quantities of sperm and induces chromosome breaks without affecting the sperm's ability to activate the eggs. However, residual paternal characteristics or chromosome fragments demonstrate the inefficiency of ionizing radiation for inducing a clean sperm inactivation (Ijiri, 1980; Chourrout and Quillet, 1982; Onozato, 1984; Allen, 1987).

Ultraviolet irradiation has been used extensively recently because it is easy to use anywhere, inexpensive and safer to apply. Furthermore, UV irradiation results in no residual fragments, in contrast to gamma irradiation (Thorgaard, 1983; Chourrout, 1984, 1986; Ihssen *et al.*, 1990; Myers *et al.*, 1995a). UV irradiation of spermatozoa acts to produce cyclobutane-type dimers between adjacent pyrimidines on the same DNA strand resulting in almost total degradation of the DNA (Ijiri and Egami, 1980). However, the low penetrating power of UV light makes the treatment of large volumes of sperm more difficult compared to gamma radiation sources. Therefore, for UV the intensity of irradiation and duration and standardisation of sperm concentration have to be optimised for successful production of haploid gynogens. Identification of a saline solution, which

can immobilise sperm, may help during the process of counting, diluting, and irradiation of sperm.

Chemical treatments such as dimethylsulphate, DMS, (Chourrout, 1986) toluidine blue, and ethyleneurea have also proven effective for the DNA inactivation of spermatozoa. However, supernumary chromosome fragments were reported by Chourrout (1986) in DMS treatments of rainbow trout sperm.

Since haploid gynogenetic embryos are grossly abnormal at hatching, some treatment is needed to produce diploid gynogenetic animals. Although there is no strong evidence for frequent spontaneous diploidization of eggs fertilised with denucleated sperm, it has been exceptionally reported in weatherfish, *Misgurnus fossilis* (Ramashov *et al.*,1960: cited in Ihssen *et al.*, 1990), common carp, *Cyprinus carpio* (Nagy *et al*, 1978), grass carp, *Ctenopharyngodon idella* (Stanley, 1976) and rainbow trout (Purdom *et al.*, 1985). Exclusion of the male genome has been observed in crosses between species in the hybrid plaice (*Pleuronectes platessa*) X halibut (*Hippoglossus hippoglossus*) (Purdom and Lincoln, 1974: cited in Ihssen *et al.*, 1990), common carp X crucian carp, *Carassius carassius*, (Golovinskaya *et al.*, 1963: cited in Ihssen *et al.*, 1990) and grass carp X common carp, (Stanley, 1976).

There are four possible mechanisms by which diploidy could be re-established during parthenogenesis: (i) suppression of endomitosis (ii) retention of the first polar body from meiosis I, (iii) retention of the second polar body from meiosis II and (iv) inhibition of first cleavage. According to Ihssen *et al.* (1990), all fish egg completes first meiotic division before ovulation, therefore only the 2nd meiotic and first mitotic divisions can be manipulated. In molluscs, however, the unfertilised egg still has to go

through both 1st and 2nd meiotic divisions when it is released providing a wider possibility for ploidy manipulation (Beaumont and Fairbrother, 1991). Early shocks prior to the loss of the second polar body can cause retention of the second polar body and produce meiotic gynogenesis, whereas with late shocks 2nd meiotic division is completed and these prevent the first mitotic cleavage and produce mitotic gynogenetic individuals. Three types of treatments are used in fishes : (i) long cold shocks, (ii) short heat shocks and (iii) short hydrostatic pressure shocks. All treatments have a destructive effect on microtubules and thus inhibit chromosome division. Each shock tested may be characterised according to three parameters; its temperature or pressure (intensity) level, its start time and its duration. Hydrostatic pressure shocks inhibit the anaphase stages of cell division by disrupting the metaphase spindle (Onozato, 1984; Hussain, 1996). On the other hand, cold and heat shocks alter different cell mechanisms. Heat shocks either permit or inhibit the entire disjunction by denaturing the spindle apparatus that plays a role in the migration of all chromosomes while the cold shocks inhibit anaphase II. Both of these can inhibit the second meiotic division (Valenti 1975; Chourrout, 1986; Diter *et al.*, 1993). Heat shock is the easiest to apply requiring facilities for pre-incubation at one temperature before shocking at a higher and more critical temperature. This can be achieved by using two water baths, which enables precise control of temperature. Cold shock is also easy to apply. However, apparatus for cooling water is more expensive than that used for heat shocks. The use of hydrostatic pressure shock requires purpose-built apparatus so is likely to be the most expensive and more skill may be required.

Since 1960 meiotic gynogenesis has been successfully induced in many fish species. Table 2.1. shows published studies reporting meiotic gynogenesis on different fish species.

Table 2.1. Published studies reporting meiotic gynogenesis on different fish species.

Scientific name	Common name	References
Cyprinus carpio	Common carp Ornamental carp	Nagy and Csanyi, 1982; Hollebecq *et al.*, 1986; Komen *et al.*, 1988; Sumantadinata *et al.*, 1990; Cherfas *et al.*, 1990
Ctenopharyngodon idella	Grass carp	Cassani and Caton, 1985
Catla catla *Labeo rohita* *Cirrhinus mrigala*	Indian major carps	John *et al.*, 1984, 1988
Pagrus major	Red sea bream	Sugama *et al.*, 1990
Oncorhynchus mykiss	Rainbow trout	Chourrout and Quillet, 1982; Thorgaard *et al.*, 1983; Chourrout, 1984
Oncorhynchus kisutch	Coho salmon	Refstie *et al.*, 1982
Danio rerio	Zebra fish	Streisinger *et al.*, 1981
Puntus gonionotus	Silver barb	Pongthana *et al.*, 1995
Silurus glanis	European catfish	Krasznai and Marian, 1987
Clarias gariepienus	African catfish	Volckaert *et al.*, 1994, 1997
C. macrocephalus	Thai walking catfish	Na-Nakorn, 1997
Misgurnus anguillicaudatus	Loach	Arai *et al.*, 1993
Plecoglossus altivelis	Ayu	Taniguchi *et al.*, 1988

Table 2.1. continued

Scientific name	Common name	References
Oreochromis species	Tilapias	Chourrout and Itskovich, 1983; Penman *et al.*, 1987; Don and Avtalion, 1988; Varadaraj, 1990; Mair *et al.*, 1991a, b; Hussain *et al.*, 1993 Perruzzi *et al.*, 1993

Suppressing the first mitotic division of gynogenetically developing eggs or normal developing eggs by applying late heat, cold or pressure shocks can produce completely homozygous individuals (mitotic gynogens) or tetraploid individuals, respectively. The intensity of temperature or pressure shock required to suppress the first mitotic division is the same or close to the level for inhibiting meiotic division. Table 2.2. shows the summary of methods used with survival rates in mitotic gynogenesis research.

2.1.2. Tetraploidy

Another possible consequence of the disruption of the first mitotic division is tetraploidy, if done after normal fertilisation. The potential applications of tetraploids are in their increased heterozygosity and growth potential, the possibility to generate large numbers of sterile triploid progeny in tetraploid X diploid matings, in the production of diploid spermatozoa for cryopreservation and their usage in androgenetic production

Table 2.2. Summary of methods used for androgenesis and mitotic gynogenesis research. %R: Relative to controls, D: Duration of treatment, I: Interval between fertilisation and start of treatment, τ_0: the time interval between the first appearance of furrow of the first and second (or the second and the third) cleavage division, a. f.: After fertilisation, Y. S. R.: Yolk sac resorption stage

Species	Reference	Source of Irradiation	Treatment	Survival
Rainbow trout	1 Chourrout, 1984	^{60}Co	Pressure: 492 $kgcm^{-2}$ D: 3 min I: 5 h 50 min	Mitotic gynogenetic Hatching: 8.2%
	2 Lou and Purdom, 1984	UV	Pressure: 562 $kgcm^{-2}$ D: 10 min I: 7h 30 min-8 h 30 min	Mitotic gynogenetic No production
	3 Purdom *et al.*, 1985	UV	Heat: 28°C D: 10 min I: 4-5 h	Mitotic gynogenetic Alevin stage: 1-7%
	4 Parsons and Thorgaard, 1985	^{60}Co	Pressure: 598-633 $kgcm^{-2}$ D: 1-3 min I: 5 h 45 min	Androgenetic diploid Hatching: 32.5-38.9% (%R)
	5 Scheerer *et al.*, 1986	^{60}Co	Pressure: 633 $kgcm^{-2}$ D: 3 min I: 5h 45 min	Androgenetic diploid Hatching: 7.2-7.9% (%R) Feeding: 4.7-4.8% (%R) from inbred sperm source Hatching: 8.8-9.5% (%R) Feeding: 5.6-6.8% (%R) from outbred sperm source

Table 2.2. continued

Species	Reference	Source of Irradiation	Treatment	Survival
	6 Thorgaard *et al.*, 1990	^{60}Co	- (Tetraploid male)	Androgenetic diploid Hatching: 11.8% Feeding: 10.3%
			Pressure: 633 $kgcm^{-2}$ D: 3 min I: 5 h 45 min	Androgenetic diploid Hatching: 1.2% First feeding: 0.6%
	7 Scheerer, *et al.*, 1991	^{60}Co	Pressure: 633 $kgcm^{-2}$ D: 3 min I: 5 h 20 min-5 h 40 min	Androgenetic diploids Hatching: 2.4-2.7% Feeding: 1.8-1.9% from outbred sperm source
				Hatching: 1.5-1.6% Feeding: 1.2-1.3% from inbred sperm source
	8 Quillet *et al.*, 1991	UV	Heat: 31.5°C D: 5 min I: 3 h 30 min	Mitotic gynogenetic Feeding: 16% (%R)
	9 Diter *et al.*, 1993	UV	Heat: 30-32°C D: 3-9 min I: 2 h-4 h 40 min	Mitotic gynogenetic Hatching: 11-15% (%R)
	10 Araki *et al.*, 1995	^{60}Co	- (Fused sperm)	Androgenetic diploid Hatching: 0.32
	11 Ueda, 1996	^{60}Co	Heat: 30°C D: 7 min I: twice at 30 sec and 3 h 30 min	Androgenetic diploid Eyed: 7.5%

Table 2.9. continued

Species	Reference	Source of Irradiation	Treatment	Survival
Brook trout	1 May *et al.*, 1988	^{60}Co	Pressure: 598 $kgcm^{-2}$ D: 3 min I: 7 h 30 min	Androgenetic diploid Eyed: 37% (%R)
Amago salmon	1 Nagoya *et al.*, 1996	^{60}Co	Pressure: 650 $kgcm^{-2}$ D: 6 min I: 7 h 30 min	Androgenetic diploid At day 30 a.f.: 0.95%
Loach	1 Suwa *et al.*, 1994	UV	Pressure: 800 $kgcm^{-2}$ D: 1 min I: 30-35 min	Mitotic gynogenetic At 6 month a.f.: 0.16%
	2 Masaoka *et al.*, 1995	UV	Pressure: 800 $kgcm^{-2}$ D: 1 min I: 35 min	Androgenetic diploid Feeding: 0.2%
	3 Arai *et al.*, 1995	UV	- (Natural tetraploids)	Androgenetic diploid Feeding: 5-9.3%
Carp	1 Nagy, 1987	^{60}Co	Heat: 40.13°C D: 2 min I: 25-75 min	Mitotic gynogenetic Low
	2 Grunina *et al.*, 1990	X ray	Heat: 40.5-41°C D: 2-3 min I: (τ_0) 1.7-1.9	Androgenetic diploid At 24 h a.f.: 9%
	3 Bongers *et al.*,1994	UV	Heat: 40°C D: 2 min I: 26-30 min	Androgenetic diploid Hatching: 8.6-19.3% At 24 days a.f.: 6.2-15.8%

Table 2.9. continued

Species		Reference	Source of Irradiation	Treatment	Survival
Zebra fish	1	Streisinger, *et al.*, 1981	UV	Pressure: 562 $kgcm^{-2}$ with 2% ether D: 5 min 30 sec I: 22 min 30 sec	Mitotic gynogenetic Maturity: 20%
				Heat: 41.4°C D: 2 min I: 10-13 min	Mitotic gynogenetic Maturity: 10-20%
	2	Corley-Smith *et al.*, 1996	X ray	Heat: 41.4°C D: 3 min 30 sec I: 13 min	Androgenetic diploid Maturity: 2%
Ayu	1	Taniguchi *et al.*, 1988	UV	Pressure: 600-700 $kgcm^{-2}$ D: 6 min I: 70 min	Mitotic gynogenetic At 6 day a. f.: 41.8-27.1%
Siberian sturgeon	1	Grunina and Neifakh, 1991	X-ray	Heat: 37°C D: 30 min I: (τ_0) 1.6	Androgenetic diploid ?: 12.2%
Catfish	1	Bongers *et al.*, 1995	UV	Heat: 41°C D: 1 min I: 33 min	Androgenetic diploid Hatching: 10.5%
Muskellunge	1	Lin and Dabrowski, 1998	UV	Heat: 31°C D: 9 min I: (τ_0) 1.4	Mitotic gynogenetic Low

Table 2.9. continued

Species		Reference	Source of Irradiation	Treatment	Survival
Blue tilapia	1	Marengoni and Onoue, 1998	UV	Heat: 41.6°C D: 5 min I: 27 min 30 sec	Androgenetic diploid Hatching: 0.5% (%R)
Nile tilapia	1	Mair et al., 1987	UV	Heat: 41.1°C D: 3 min 30 sec I: 30-35 min	Mitotic gynogenetic Pigmenting: 7.34%
	2	Hussain et al., 1993	UV	Heat: 41°C D: 3.5 min I: 30 min	Mitotic gynogenetic Y. S. R.: 2%
				Pressure: 633 D: 2 min I: 40-50 min	Mitotic gynogenetic Y. S. R.: 1.2%
	3	Myers et al., 1995	UV	Heat: 42.5°C D: 4 min I: 20-30 min	Mitotic gynogenetic Y. S. R.: 0.5-10.64% (%R)
				Heat: 42.5°C D: 3-4 min I: 22 min 30 sec-30 min	Androgenetic diploid Y. S. R.: 0.4-5.3% (%R)
	4	Marengoni and Onoue, 1998	UV	Heat: 41.6°C D: 5 min I: 27 min 30 sec	Androgenetic diploid Hatching: 0.75% (%R)
	5	Present study	UV	Heat: 42.5°C D: 3 min 30 sec I: 25 min	Androgenetic diploid Pigmentation: 10.69% (%R) Hatching: 2.03% (%R) Y. S. R.: 0.07% (%R)

(Chourrout, 1987). Few reports are available on tetraploid fish compared to meiotic or mitotic gynogenesis. Various chemicals that interfere with mitosis, such as colchicine and cytochalasin B, have been used to induce polyploidy but with much less success. Refstie *et al.* (1977), Smith and Lemoine (1979) and Refstie (1981) used cytochalasin B at concentration of 10 μg/ml and observed polyploid mosaics and some tetraploids in rainbow trout. However, they used ploidy identification by nuclear volume measurement which is unreliable and does not readily distinguish between triploids and tetraploids, so it is uncertain whether tetraploids were actually produced. Thorgaard and Jazwin (1981) obtained 10 % tetraploid rainbow trout that survived 20 days after fertilisation using a 36°C heat shock for 1 min applied 5 h after fertilisation. Lou and Purdom (1984) attempted to produce tetraploid in rainbow trout by hydrostatic pressure treatment of 562 $kgcm^{-2}$ for 10 min with 2 % ether pre-treatment or with a heat shock of 28°C for 10 min begun 8 h after fertilisation without success. On the other hand, Chourrout (1984) reported that 100 % tetraploids with a survival rate of 30% to first feeding stage were produced by 492 $kgcm^{-2}$ pressure shocks applied 5 h 50 min after fertilisation lasting 4 min in the same fish. Diter *et al.* (1993) reported very low survival of tetraploid using a heat shock of 31°C for 5 min applied 3 h 20 min after fertilisation in rainbow trout. Induction of tetraploidy using late hydrostatic pressure of 680 $kgcm^{-2}$ in chum salmon, *Oncorhynchus keta*, and masu salmon, *O. masou*, was not successful (Yamazaki and Goodier, 1993). In brown trout, production of tetraploidy using late pressure shock of 633 $kgcm^{-2}$ for 6 min gave poor survival (Myers *et al.*, 1995b).

Tetraploidy induction was attempted by Myers (1986) by using a combination of pressure (492 $kgcm^{-2}$) and cold treatments (7.5°C) applied for 7 min just prior to cleavage and survival rates were reported as 2.4±3.5 %, 8.3±11.7 % and 1.2±1.1 %, in *O. niloticus, O. mossambicus* and their hybrid, respectively to eyed stage. Don and Avtalion (1988) obtained low percentage of tetraploidy (12% to yolk sac stage) but not beyond using cold shock treatment of 1 h at 11°C applied at 60 min after fertilisation in *O. aureus*.

2.1.3. Androgenesis

Androgenesis is a genome manipulation technique, which is the opposite of gynogenesis involving a genetically inactivated egg fertilised with normal sperm. The resulting embryo develops with entirely paternal chromosomal inheritance, without any contribution from maternal chromosomes. The egg can be inactivated successfully by gamma, X rays or UV irradiation. Haploid androgenesis has been induced using ^{60}Co in loach (Ihssen *et al.*, 1990), flounder, *P. flesus* (Purdom, 1969), masu salmon (Arai *et al.*, 1979), rainbow trout (Parsons and Thorgaard, 1985) and brook trout, *Salvelinus fontinalis* (May *et al.*, 1988). Yamazaki (1983) observed the spontaneous occurrence of haploid androgenetic embryos where the oocytes of salmonid species were overripe or in some interspecific or intergeneric crosses in which pronuclei did not fuse. Briedis and Elison (1982) induced haploid androgenetics in fertilised frog, *Rana pipiens*, eggs using pressure and deuterium oxide (D_2O) to inhibit male pronucleus movement by the distruptive effects of microtubule-specific agents on pronuclear movement.

Studies with amphibians (Gillespie and Armstrong, 1980, 1981) showed that the transparency of the amphibian egg and the fact that the egg pronucleus is oriented toward

the animal pole after fertilisation facilitated treatments with UV. However, the opacity of some fish eggs and the failure of the egg nucleus to demonstrate any particular orientation before or after fertilisation may present problems owing to the poor penetrance of UV (Thorgaard, 1983; McAndrew *et al.*, 1993). Despite these disadvantages, UV light has been successfully used in the irradiation of eggs from white sturgeon, *Acipenser transmontanus* (Kowtal, 1987), common carp (Bongers *et al.*, 1993, 1994), Nile tilapia (Myers, 1995a), loach (Arai *et al.*, 1992) and African catfish, *Clarias gariepinus*, (Bongers *et al.*, 1995).

Spontaneous androgenesis has sometimes been observed in crosses of different fish species. Stanley (1976) observed androgenetic grass carp at a low frequency, in crosses between female common carp with male grass carp. The diploids might have been generated from genetic incompatibility between the two genomes resulting in loss of the female pronucleus. Spontaneous diploidization of X-ray genetically inactivated common carp eggs fertilised with sperm of crucian carp and common carp was reported by Cherfas *et al.* (1994).

Androgenetic diploidization can be induced by suppression of the first mitotic division of the egg by means of physical shocks such as temperature and pressure as in mitotic gynogenesis or by fertilisation of inactivated eggs with diploid spermatozoa from tetraploid males if the latter can be produced. The first successful production of androgenetic diploids was reported by Gillespie and Armstrong (1980, 1981) in the Mexican axolotl, *Ambystoma mexicanum*, using heat shock (36-37°C for 10 min) or hydrostatic pressure shock (984 $kgcm^{-2}$ for 8 min) at 5.5 hrs post fertilisation. Table 2.2.

(page 41) summarised the methods used with survival rates in androgenetic research in fish.

2.1.4. Application of gynogenesis and androgenesis

The major rationale for the interest in gynogenesis has been the potential for producing inbred lines for breeding programmes and research purposes (Ihssen *et al.*, 1990; Purdom, 1969). However, meiotic gynogenetic animals are partially heterozygous for many generations due to the occurrence of recombination between chromatids during the first meiotic division of the egg (Purdom, 1969; Nace *et al.*, 1970; Hussain *et al.*, 1994). It was thought at first that little crossing-over between genes and the centromere occurs in fish chromosomes (Purdom, 1969) therefore low levels of heterozygosity were expected. However, subsequent studies showed high levels of heterozygosity at some loci in gynogenetic diploids. Gynogenetic diploids that have loci that remain heterozygous and have the same genotype as their mothers have had to have undergone recombination. This has been noted in common carp (Golovinskaya and Ramashov, 1966: cited in *Ihssen et al.*, 1990), rainbow trout (Thorgaard *et al.*, 1983; Guyomard, 1984), brown trout (Guyomard, 1986), Nile tilapia (Hussain *et al.*, 1994). Meiotic gynogenesis is a very useful tool for estimating gene-centromere recombination rates since the level of recombination is expected to be proportional to any genes distance from a centromere (Hussain *et al.*, 1994). It is also important to increase the rate of inbreeding and in gene mapping studies (Thorgaard and Allen, 1987). On the other hand, mitotic gynogenesis and androgenesis can be applied to produce completely homozygous individuals in the

first generation and clones in the second generation. Since clones are completely homozygous for every gene locus, they have potential for fixing superior genes and thus the production of new lines of fish. The inbred lines, which are supposed to be free from recessive lethal and major deleterious alleles, may be crossed to obtain a degree of heterosis for commercially important traits. Clones have been successfully produced for zebra fish (Streisinger *et al.*, 1981), medaka (Naruse *et al.*, 1985; Ijiri, 1987), common carp (Komen *et al.*, 1991), ayu (Han *et al.*, 1991) and Nile tilapia (Hussain *et al.*, 1998; Sarder, 1998) by mitotic gynogenesis. Production of androgenetic clones have been reported in amago salmon, *Oncorhynchus rhodurus* (Nagoya *et al.*, 1996) and Nile tilapia (Jim Myers personal communication of Sarder, 1998).

Both gynogenesis and androgenesis can be used to analyse the genetic basis of complex traits such as red colouration and sex determination in Nile tilapia and these techniques or combination of these techniques offer a relatively rapid method to develop new lines.

Another important use of gynogenesis and androgenesis has been in the analysis of sex determining mechanisms in fish. Producing a monosex female population by gynogenesis in fish with female homogamety was achieved in *O. niloticus* and *O. mossambicus* (Penman *et al.*, 1987, 1989; Mair *et al.*, 1991a, b), and in silver barb, *Puntus gonionotus* (Pongthana *et al.*, 1995). Hormonal sex reversal of gynogenetic females to males can ensure the production of all female populations by crossing the sex reversed neomales to normal females. Sex reversed neofemales have been used for the production of YY genotypes for production of all male populations by gynogenesis in *O. mossambicus* (Varadaraj and Pandian, 1989) and in *O. niloticus* (Scott *et al.*, 1989). On

the other hand, androgenesis should lead to the production of all male monosex population in fish with male homogamety or 50 % (YY) male with 50 % female (XX) in species with male heterogamety. In the case of the *O. niloticus*, the genotype of inbred male androgens would be YY and they can be used to produce all male progeny in the subsequent crosses with any ordinary XX females (Thorgaard, 1983; Myers *et al.*, 1995a). Hormonally sex reversed androgenetic tilapia neofemales (YY) can also be used to produce all YY male offspring crosses with YY male (Mair, 1993).

One powerful application of androgenesis is to recover genotypes from cryopreserved sperm, particularly for those which are facing extinction and threat of contamination by hybridization. Sperm cryopreservation, unlike that of eggs, is becoming relatively routine in some species and its combination with androgenesis offers a invaluable way to conserve genetic resources (Stoss, 1983; Ihssen *et al.*, 1990; McAndrew *et al.*, 1993; Mair, 1993).

2.1.5. Identification of gynogenetic and androgenetic diploids

When gynogenetic and androgenetic diploids are produced, it is important to have proof that the sperm (in the case of gynogenesis) and or the eggs (in the case of androgenesis) did not contribute genetically to the embryo. This proof may be obtained by several methods. The simplest method for gynogenesis studies is the use of irradiated sperm from a related species to trigger development (Nace *et al.*, 1970; Stanley, 1976). In this case any paternal inheritance might be recognised by inviable hybrids,

morphologically recognisable hybrids and biochemically recognisable hybrids (Thorgaard, 1983).

Mendelian visible markers particularly colour have been used for confirmation of all maternal (Streisinger *et al.*, 1981; Thorgaard *et al.*, 1985; Varadaraj, 1990) and all paternal inheritance (Parsons and Thorgaard, 1985; Grunina et al., 1990; 1991; Bongers *et al.*, 1994; Myers *et al.*, 1995a) in mitotic gynogenesis and and androgenesis, respectively. However, the availability of such visible markers is rare in most species and the interference of chromosome fragments which can be generated by ionizing irradiation (Chourrout and Quillet, 1982; Parsons and Thorgaard, 1985) might not be detected by using colour markers.

The biochemical markers, screened by starch gel electrophoresis are more useful than morphological or colour markers for detection of maternal (androgenesis) and paternal (gynogenesis) transmission or differentiation of meiotic and mitotic gynogens which is important if there is an overlap in the induction windows for these two types of gynogens due to asynchronous zygote development up to first cleavage (Mair, 1993). Allozyme polymorphism have confirmed all maternal inheritance in mitotic gynogenesis (Purdom *et al.*, 1985; Mair *et al.*, 1987a; Taniguchi *et al.*, 1988; Hussain *et al.*, 1993; Suwa *et al.*, 1994) and all paternal inheritance in androgenesis (Parsons and Thorgaard, 1985; Scheerer *et al.*, 1986; May *et al.*, 1988; Arai *et al.*, 1995; Myers *et al.*, 1995a; Marengoni and Onoue, 1998).

Nowadays, DNA-level markers provide very accurate parentage assessment. Amongst them, microsatallite markers have a great potential since these loci appear to be highly abundant and dispersed throughout the genome (O'Connell and Wright, 1997).

The drawbacks of DNA-level markers as compared with biochemical or protein-level markers are that they are relatively expensive in terms of materials and labour and the procedure for visualisation of marker phenotypes are more demanding technically. Since the present study aimed to use multilocus DNA fingerprinting technique for confirmation of all paternal inheritance, more detail will be given on this subject in the next section.

2.1.5.1. DNA Fingerprinting

Most genes in eukaryotes consist of coding sequences (exons), non-coding regions (introns) and flanking region (enhancer and promoter). When a gene of a higher organism is to be expressed, it is transcribed to yield an RNA copy of the exon and intron sequences. This primary RNA transcript is then processed to yield messenger RNA (mRNA) which contains only exon sequences. Other non-coding DNA which is not used in RNA transcription is present in various numbers of copies called highly repetitive DNA, with no known function (Krawczak and Schmidtke, 1994). In total genomic DNA, about one-third consists of repetitive sequences. According to the organisation and degree of repetition of this type of DNA in the genome, it can be divided into two classes; interspersed repeat sequences and tandem repeat sequences. In the interspersed repeats, the repetitive DNA sequences are scattered at multiple sites throughout the genome. Interspersed repeat sequences can be either short interspersed elements (SINEs), which are less than 500 base pairs long and present as many as a million times, or long interspersed element (LINEs), which are around 6400 base pairs long and present between 3000 and 40000 times. Repetitive sequence elements, which are arranged in tandem, are known as satellite, minisatellite and microsatellite sequences. Satellite DNA,

which is dispersed over almost the entire genome, is composed of very high copy number repetitions of a basic sequence (between 10^3-10^7 per locus). The length of the repeat unit is usually 100-300 base pairs. Minisatellites consist of shorter repetitive DNA sequence, usually 10-60 base pairs and show a lower degree of repetition (between 10-10^3 per locus). Microsatellites comprise very short (between 1 and 10 base pairs) repetitive sequences with a lowest degree of repetition (between 10-10^2) and are called "simple sequences" (Klug and Cummings, 1996).

Satellites, minisatellites and microsatellites can be highly variable and thus form excellent tools for population genetic, parentage assessment and genome mapping studies. Their variability is most often due to particular arrays on a given chromosome having different repeat numbers in different individuals. Thus, they form allelic variants and for a number of mini and microsatellites almost every individual is heterozygous. Polymorphism created by such elements is termed variable number of tandem repeat (VNTR) polymorphism. Polymorphism due to variation in the number of elements with in a given array is thought to be generated during DNA replication, for example by the mutational process of slipped strand mispairing and by unequal crossovers, which means that cleavage and reunion of the two strands involved does not occur at allelic sites, during meiosis. As a result of unequal crossover, a DNA sequence lying adjacent to the non-identical break points will be found in duplicate on one recombinant chromatid but will be lacking from the other (Jarman and Wells, 1989; Avise, 1994; Krawczak and Schmidtke, 1994). Repetitive DNA sequences have been turned to advantage in terms of providing individual specific genetic markers. The term "DNA fingerprinting" was first introduced by Jeffreys *et al.* (1985) using Southern blot analysis of hypervariable DNA sequences. The DNA probes namely 33.6 and 33.15 originally employed by Jeffreys *et*

al. (1985) were isolated from a myoglobin intron in human. The DNA profile can be generated by cleaving total genomic DNA on either side of the minisatellite, not within the repeated sequence, by means of a particular restriction enzyme.

The length of a restricted fragment will depend on the number of repeats between sites and generate restriction fragment length polymorphism (RFLP). The DNA fragments are separated by agarose gel electrophoresis, transferred to a nylon membrane and finally hybridised to 10-15 base pairs long conserved core sequences of the repeat units in the human genome, revealing band profiles distinguishing all individuals except monozygotic twins. The complexity of the DNA profiles depends on the number of loci recognised by the hypervariable probe. In "multilocus fingerprinting", the probe hybridises to several loci scattered among the genomic DNA. Since spontaneous *de novo* mutation is rare (Jeffreys *et al.*, 1987, 1988) and bands composing the fingerprinting pattern are inherited in a Mendelian fashion, each of the bands in an individual's DNA fingerprint profile must originate from either its biological father or mother. In "single locus DNA fingerprinting" the hypervariable probe hybridises to only one locus and the individual DNA profile shows either one or two bands depending on whether such an individual is homozygous or heterozygous.

Multilocus DNA fingerprinting is a powerful technique for genetic studies. It has been successfully applied for paternity and maternity analysis in humans (Jeffreys *et al.*, 1986), in birds (Burke and Bruford, 1987; Burke *et al.*, 1989, 1991), in forensic studies (Bär and Hummel, 1991), genetic variability within and between populations in California Channel Island fox (Gilbert *et al.*, 1990), in naked mole-rat (Reeve *et al.*, 1990) and linkage analysis in human (Jeffreys *et al.*, 1986). In aquaculture, DNA fingerprinting can be used for the identification of individuals, construction of pedigree,

population analysis (Hallerman and Beckman, 1988), estimation of inbreeding rates in commercial broodstocks (Doyle and Talbot, 1986; Eknath and Doyle, 1990) and family identification without using tags, especially for small fish. Another advantage of using DNA fingerprints has been proposed to monitor the absence of paternal genomic contribution in fish produced by meiotic or mitotic gynogenesis, as well as to measure the degree of homozygosity after meiotic gynogenesis (Chourrout, 1986). DNA fingerprinting has been used to verify succesful gynogenesis and androgenesis in fish (Carter *et al.*, 1991; Han *et al.*, 1992; Takagi *et al.*, 1995; Eenennaam *et al.*, 1996; Nagoya *et al.*, 1996; Young *et al.*, 1996; Sarder *et al.*, in press).

2.1.6. Objectives

The objectives of the present study are:

1. Optimisation of treatment parameters to induce diploid androgenesis in *O. niloticus*,

2. Production of homozygous red and blond YY males by androgenesis and thus fixing of both traits in a single line of *O. niloticus*.

3. Production of homozygous red and blond YY neofemales by oral administration of feminisation hormone (Diethylstilbestrol) to obtain all homozygous red and blond YY male population by crossing the androgenetic YY neofemales to androgenetic YY males in *O. niloticus*.

4. Further investigation of sex and colour mechanisms of *O. niloticus*.

2.2. Materials and methods

2.2.1. Experimental fish stocks

The tilapia species, *Oreochromis niloticus* L. used in the experiments described and discussed in this thesis came from the Tilapia Reference Collection at the Institute of Aquaculture, University of Stirling, Scotland. They were originally obtained from Lake Manzala, Egypt in 1979 and have been shown to be a pure species by electrophoresis (McAndrew and Majumdar, 1983). Stirling red and blond *O. niloticus* from the same fish stock of the Institute of Aquaculture also were used. The origin of these red and blond fish is described by Scott *et al.* (1987), McAndrew *et al.* (1988) and Hussain *et al.* (1994).

2.2.1.1. Rearing and stocking facilities

All fish were reared in recirculating fresh water systems except those which were fed with hormone treated food, which were kept in static systems. The systems were maintained in the tropical aquarium facilities at the Institute of Aquaculture. Lighting in all the systems was adjusted by an automatic timer to 12 hour light and 12 hour dark. The water temperature was maintained 28±1°C. All precipitated solid waste in bottom settling tanks was siphoned out and biological filter trays cleaned once a week.

Individual female broodstock were kept in partitioned glass tanks of 120 cm X 44 cm X 30 cm (Figure 2.1). Each glass tank incorporated two or three vertical dividers (depending on fish size), made of translucent Perspex (0.5 cm thick). Therefore, each tank created three or four separate "holding spaces" in which a female's maturity state

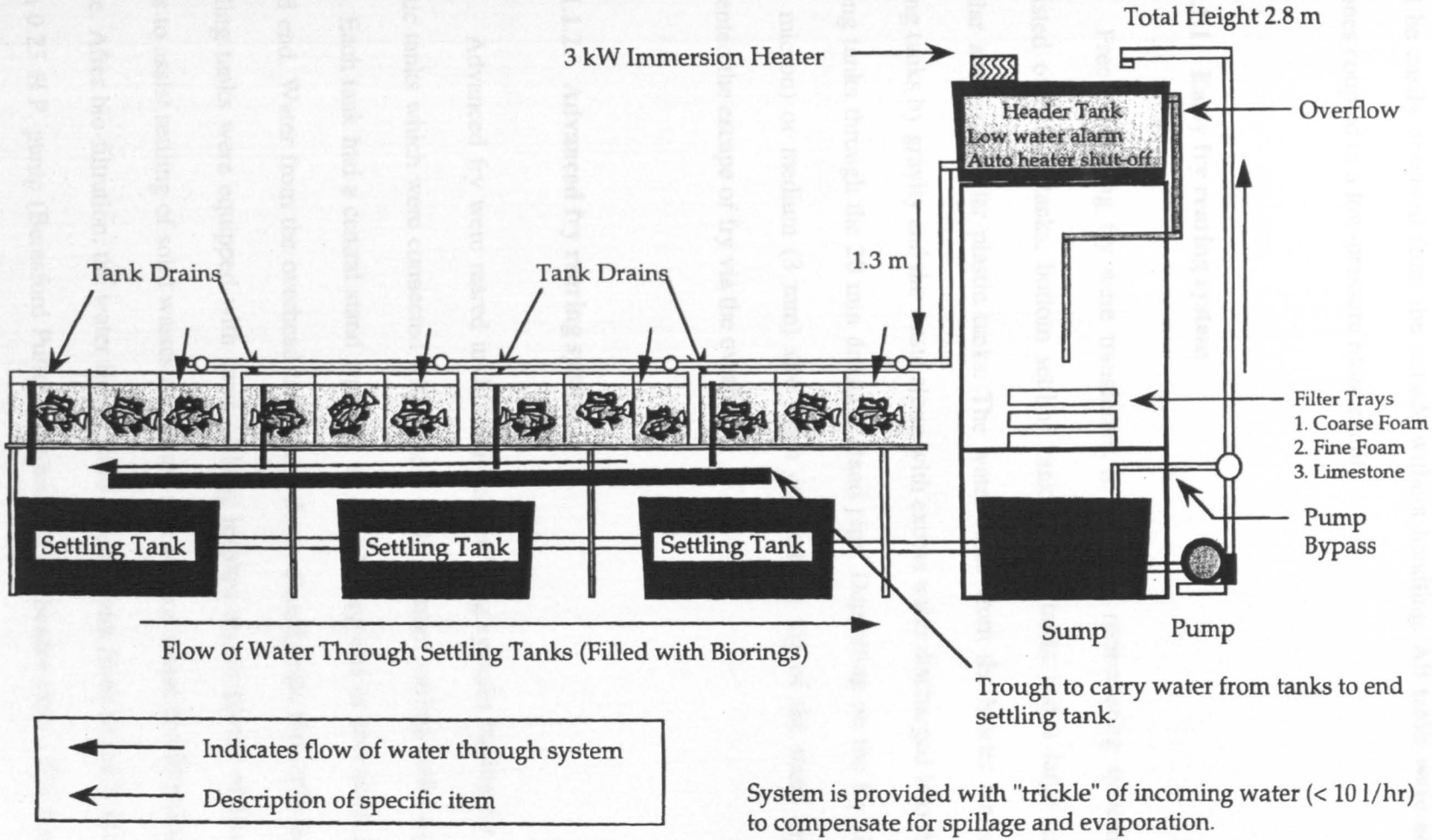


Figure 2. 1. Schematic diagram of tilapia broodstock recirculating system (From McAndrew *et al.*, 1995).

could be easily observed from the outside without handling. All tanks were aerated by airstones coupled to a low-pressure blower unit.

2.2.1.1.1. Early fry rearing system

Free swimming fry were transferred to different recirculating systems, which consisted of header tanks, bottom settling tanks, pump tanks and a large number of circular and rectangular plastic tanks. The water came from the header tanks into the rearing tanks by gravity and the waste along with excess water discharged into the bottom settling tanks through the 20 mm drainage stand pipe. Depending on the fry size, small (500 micron) or medium (3 mm) size mesh covered the top of the stand pipe which prevented the escape of fry via the overflow.

2.2.1.1.2. Advanced fry rearing system

Advanced fry were reared in different recirculating systems having 30 l circular plastic tanks which were connected with header tanks, bottom settling tanks and a pump tank. Each tank had a central stand pipe and an inlet having one or two small jets at the blind end. Water from the overhead tank came down directly into the tanks by gravity. Settling tanks were equipped with rows of long brushes and/or plenty of floating bio-rings to assist settling of solid wastes and to act as a surface where bio-filtration can take place. After bio-filtration, the water flowed into a pump tank from where it was pumped by a 0.25 H.P. pump (Beresford Pump Ltd.) back to the header tanks. The excess water

from the header tank overflowed and passed through trays full of limestone, shell, and a series of fine synthetic filters back into the pump tank.

2.2.1.1.3. Stocking system

The stocking system consisted of 2 x 180 l header tanks, 2 x 180 l bottom settling tanks, a 180 l pump tank and 16 fibre glass tanks (100 cm x 100 cm x 30 cm). These fibre glass tanks were equipped with a central stand pipe of 40 mm and arranged in double rows of 8 tanks each on a two tier system.

2.2.1.2. Water quality

To monitor water quality, ammonia, nitrate, nitrite and pH levels were measured with a Dry-tab master test kit once a week.

2.2.1.3. Feeding

All sizes of fish were fed with commercial trout feed (Trouw Aquaculture Nutrition, Russhive, UK.) The proximate composition of different feeds is presented in Table 2.1. The early and late fry were fed with micronised no. 2 or 3 pellet which was sieved to give a 0.25-1.0 mm particle size using a Moulinex coffee grinder and fed 3-4 times in a day *ad libitum*. The advanced fry and fingerlings (10-40 g size) received no. 3 sized food at rate of 3-7.5% body weight, 3 times a day. The feeding ration was reduced to 2-3% body weight with an increased pellet size of no. 4 for 40 g up to 80 g and no. 5 for 80 g fish up to broodstock size.

Table 2. 3. Proximate composition of different feeds used in rearing of experimental fish (% dry matter basis) (Source –Trouw Aquaculture)

Parameters	Feed no. 2	Feed no. 3	Feed no. 4	Feed no. 5
Protein	54.0	54.0	40.0	40.0
Oil	15.0	15.0	8.0	8.0
NFE	12.0	12.0	29.5	29.5
Fibre	1.0	1.0	4.5	4.5
Ash	10.0	10.0	10.0	10.0
Moisture	8.0	8.0	8.0	8.0

2.2.2. Anaesthesia

To minimise handling stresses during experimental studies (breeding, sampling, tagging, fin clipping and blood collection) the fish were anaesthetised individually with benzocaine (ethyl 4- aminobenzoate, Sigma. Chem. Co., Dorset, UK.) at a concentration of 1:10000. As benzocaine is not water soluble, a stock solution was first prepared by dissolving benzocaine powder at 10% w/v in ethanol. The fish were immersed in the diluted solution until they lost equilibrium and opercular movement stopped; in this condition they could be handled for up to 5 minutes. After anaesthesia, the fish were transferred to their tanks with rapid water flow and aeration. Generally, fish recovered within 2-3 minutes.

2.2.3. Tagging of fish

To identify each fish, they were tagged at 2-3 months old with Passive Integrated Transponder tags (Avid. Inc. California, USA). These have a nine digit code number and that can be read by an Avid tag reader (Power tracker II). After anaesthetising the fish, a small dorso–ventral incision was made just above the anus with a sharp sterile scalpel blade. The tag was inserted into the body cavity and a small amount of Orahesive Protective Powder (E.R. Squibb and Sons Ltd., Middlesex, UK) was spread over the incision to assist healing and prevent infection.

2.2.4. Fish breeding, stripping and fertilisation of eggs

Selected sexually mature females and males were transferred from broodstock tanks to a series of glass aquaria (120 cm x 44 cm x 30 cm) connected to a recirculating water system (Fig. 2.1). Generally three tagged females were accommodated in one tank and separated by sheets of Perspex. Aeration was provided in each tank by a 15 cm air stone connected to a central blower system.

Under aquarium conditions, mature females of *O. niloticus* spawn at approximately 2-6 weeks intervals. Females which are ready to spawn have a swollen urogenital papilla and show pre-spawning behaviour such as nest building and cleaning. After anaesthetising the female, the eggs were collected by applying gentle downward pressure with the fingers from below the pectoral fin to the genital opening of the fish. The eggs were collected in a clean, sterile Petri dish (100 mm in diameter) and were washed carefully with water from the recirculating system several times until ovarian

fluid and any blood were removed. After stripping, the eggs were sub-divided into a number of batches, as the experimental design required. Milt was also stripped from males in a similar way to egg collection using a glass capillary tube to collect the milt (BDH). Milt was then put into a clean 1.5 ml microtube and stored at 4°C until use. Milt contaminated with water and urine was rejected.

Eggs were fertilised *in vitro* by mixing the milt with "dry" eggs and then 10-20 ml of aquarium water was added. The fertilised eggs were left in the Petri dish for 2-30 minutes for water hardening, washed, and transferred to downwelling incubators for further development.

2.2.4.1. Incubation of eggs

A series of 750 ml round bottomed plastic soft drink bottles were used for egg incubation. The jars were connected to warm water (28±1°C) recirculating system (Figure 2.2). Water in the system passed by gravity from a 125 l header tank through a 30 W UV sterilisation unit (flow rate 20 ml/min, UV dosage 62000μW.sec.cm^{-2}) then through 20 mm PVC pipe to the incubation jars. Each of these had a 1 ml disposable pipette with 1 mm diameter connected to the main water supply by a small airline tap to control the water flow so as to ensure gentle movement of eggs at all times (Rana, 1986). The waste water was discharged into a 180 l bottom settling tank via two filter trays filled with crushed cockle shells, positioned just above the settling/pump tank. The shell filters were cleaned weekly to ensure good maintenance of the pH of the system.

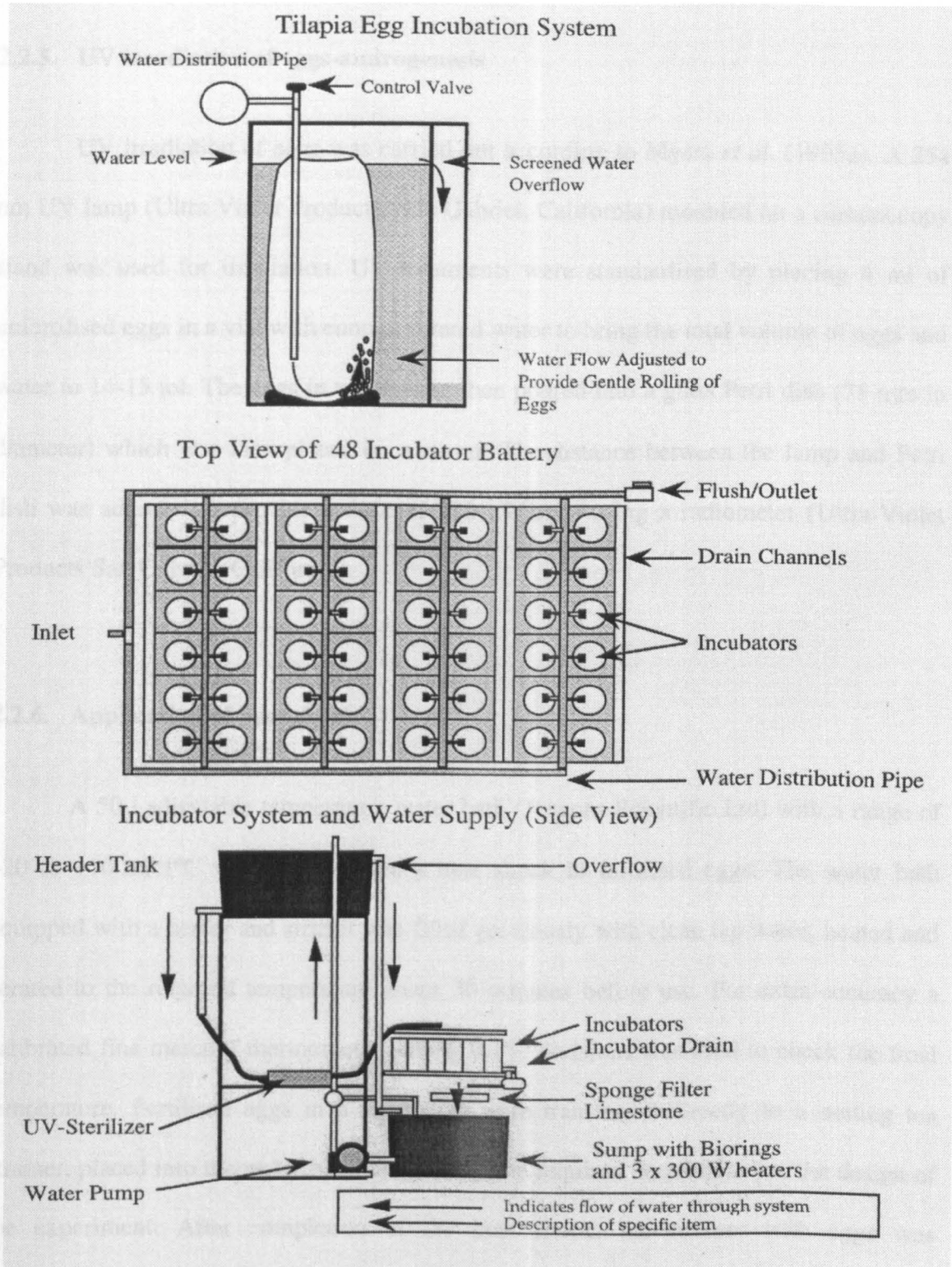


Figure 2. 2. Diagram of egg incubation bottles and Water recirculating system (From McAndrew *et al.*, 1995)

2.2.5. UV irradiation of eggs-androgenesis

UV irradiation of eggs was carried out according to Myers *et al.* (1995a). A 254 nm UV lamp (Ultra Violet Products, San Gabriel, California) mounted on a camera copy stand was used for irradiation. UV treatments were standardised by placing 4 ml of unfertilised eggs in a vial with enough filtered water to bring the total volume of eggs and water to 14-15 ml. The eggs in water were then poured into a glass Petri dish (75 mm in diameter) which was then placed on a stirrer. The distance between the lamp and Petri dish was adjusted to provide a dose of 150 uW/cm^{-2} using a radiometer (Ultra-Violet Products San Gabriel, California).

2.2.6. Application of heat shock

A 50 l adjustable temperature water bath (Jencons Scientific Ltd) with a range of –20 to +100±0.1°C was used to give a heat shock to fertilised eggs. The water bath equipped with a heater and strirrer was filled previously with clean tap water, heated and aerated to the required temperature about 30 minutes before use. For extra accuracy a calibrated fine mercury thermometer having 0.1°C divisions was used to check the final temperature. Fertilised eggs in a Petri dish were transferred directly to a netting tea strainer, placed into the water bath, and left for the required duration as per the design of the experiment. After completion of the heat shock, the strainer with eggs was immediately placed back into water at a temperature of 28±1°C and transferred to the incubator.

2.2.7. Feminisation

Application of the optimum feminisation treatment of diploid androgenetic *O. niloticus* was carried out according to Mair and Santiago (1994) by oral administration of diethylstilboestrol (DES). The DES-treated food was prepared by the alcohol evaporation method, to give a concentration of 1000 $mg.kg^{-1}$ of finely sieved no 2 or 3 food (Table 2.1) The required amount of DES hormone was weighed and dissolved in ethanol (50 ml of ethanol was used per 100 g of food). The dissolved hormone was poured onto the food in a fume cupboard and mixed frequently by a spatula to ensure even distribution of the ethanol/DES solution. The food was left in a fume cupboard to dry, then stored in airtight containers at 4°C in a refrigerator.

2.2.8. Fish sexing

The acetocarmine staining method (Guerrero and Shelton, 1974) was used to determine the sex of juvenile fish (<2 g size, about 1.5-2 months old). The stain was prepared by adding 0.5 g of acetocarmine into 100 ml of 45% acetic acid and was then boiled for about 4 minutes in fume cupboard. After cooling, the mixture was filtered using a syringe filter (Nalgene, (Europe) Ltd., Herefordshire, UK.). Fish were killed by using an overdose of benzocaine and dissected to take out the tiny thread-like gonads. The gonads were then checked under a microscope by placing them individually on clean glass slides with a drop of acetocarmine stain and squashing with a cover slip.

Examining the urogenital papilla easily differentiated the gender of the fish larger than 20-30 g. Male *O. niloticus* have a single common posterior opening whereas females have separate urinary and oviduct openings (Chervinski, 1983).

2.2.9. Incubation of eggs and checking survival rates

Both treated and untreated eggs (control) for all experiments were separately incubated (Section 2.2.4.1). The embryos in each batch were checked and counted at four development stages: morula 6-8 hours (hrs) after fertilisation (a.f); pigmentation 45-50 hrs. a.f; hatching 80-90 hrs. a.f. and yolk sac resorption 9-11 days a.f. Survival was calculated as: (Number of embryos surviving at a given development stage / total number of eggs) x 100.

2.2.10. Karyological examination for ploidy determination

Fish metaphase chromosome spreads were prepared from newly hatched or one day old post hatched larvae following some minor modification of the original procedures described by Kligerman and Bloom (1977), Chourrout and Itskovich (1993) and Chourrout (1986). Embryos were placed in a Petri dish containing 0.002-0.005% colchicine solution and left for 5-6 hrs at 25°C. Following that, they were transferred to a chilled 0.7% NaCl solution and the head and yolk sac removed under a binocular microscope using a pair of surgical needles. The dissected tissues were kept in distilled water for 8-12 mins then fixed in 3:1 methanol: acetic acid. Fixing solution containing embryonic tissue can be kept from 30 mins up to 6 weeks at 4°C. Embryonic tissues were

removed from the fixative and excessive fixative blotted off with tissue. They were then placed in a 5 mm diameter and 7 mm deep flat-bottomed hole made in a 10 mm thick Perpex block, with 2-3 drops of 50 % acetic acid. A 3 mm diameter glass rod was used to grind the tissue for 1 min. and the cell suspension was then left for 10 min. It was then taken up into a capillary tube and dropped from a height of 30-40 cm onto a slide on a hot plate (45°C). To make a fine circle from the drop, most of the remaining fluid was sucked back into the capillary tube within 8-10 sec. The process was repeated to produce 2 or 3 rings per slide. After 1 min, slides were removed from the hot plate and air dried before staining with Giemsa (prepared in 0.01 M phosphate buffer, pH : 7.0) for 20 min. The slides were then rinsed in distilled water to remove excess stain, air dried and placed in xylene for 10 min, dried again and finally mounted with DPX (BDH Ltd.). Chromosome spreads were identified around the edge of the circle under X 40 magnification and the number of chromosomes counted under X 100 (oil immersion) magnification using an Olympus compound microscope.

2.2.11. Progeny testing and determination of sex ratio

The sex of diploid androgenetic fish (>20-30 g) was determined by checking the urogenital papilla. Progeny testing of putative androgenetic YY males was carried out by crossing with an ordinary female while DES treated androgenetic YY neofemales were crossed to XX neomales. The sex ratios of the resultant offspring were determined by acetocarmine squash (Section 2.2.8).

2.2.12. Experimental design of androgenesis in tilapia

Androgenesis was carried out according to Myers *et al.* (1995a). Eggs were collected in a clean Petri dish by gently stripping ovulated females. Oocyte denucleation was accomplished by UV irradiation with a dose of 150μm.cm^{-2} (Section 2.2.5). Mitotic inhibition to "diploidize" haploid androgenetic zygotes was performed using a heat shock treatment (Section 2.2.6). Eggs were poured directly into plastic tea strainers immersed in a temperature-controlled water bath at 42.5° C. Following treatment, the eggs were put back into the incubation system. Incubation temperature was maintained at 28±1°C throughout the experiment. All treated, untreated control and UV treated control groups were checked and counted at 4 development stages (Section 2.2.9) (Fig. 2.3).

2.2.12.1. Experimental design for optimisation of UV duration time

Optimisation of UV duration time was carried out by irradiating 6 batches of eggs with UV light for 2,4,6,8,10 or 12 minutes and fertilising with sperm from blond tilapia males. This colour pattern was first reported by Scott *et al.* (1987) and can be used as a visual marker to indicate the successful production of the haploid androgenetic fish because of a recessive "blond" skin pigmentation marker. A portion of the eggs was retained as a control group and fertilised with sperm from the same blond male. Four different females and one blond male were used for this experiment.

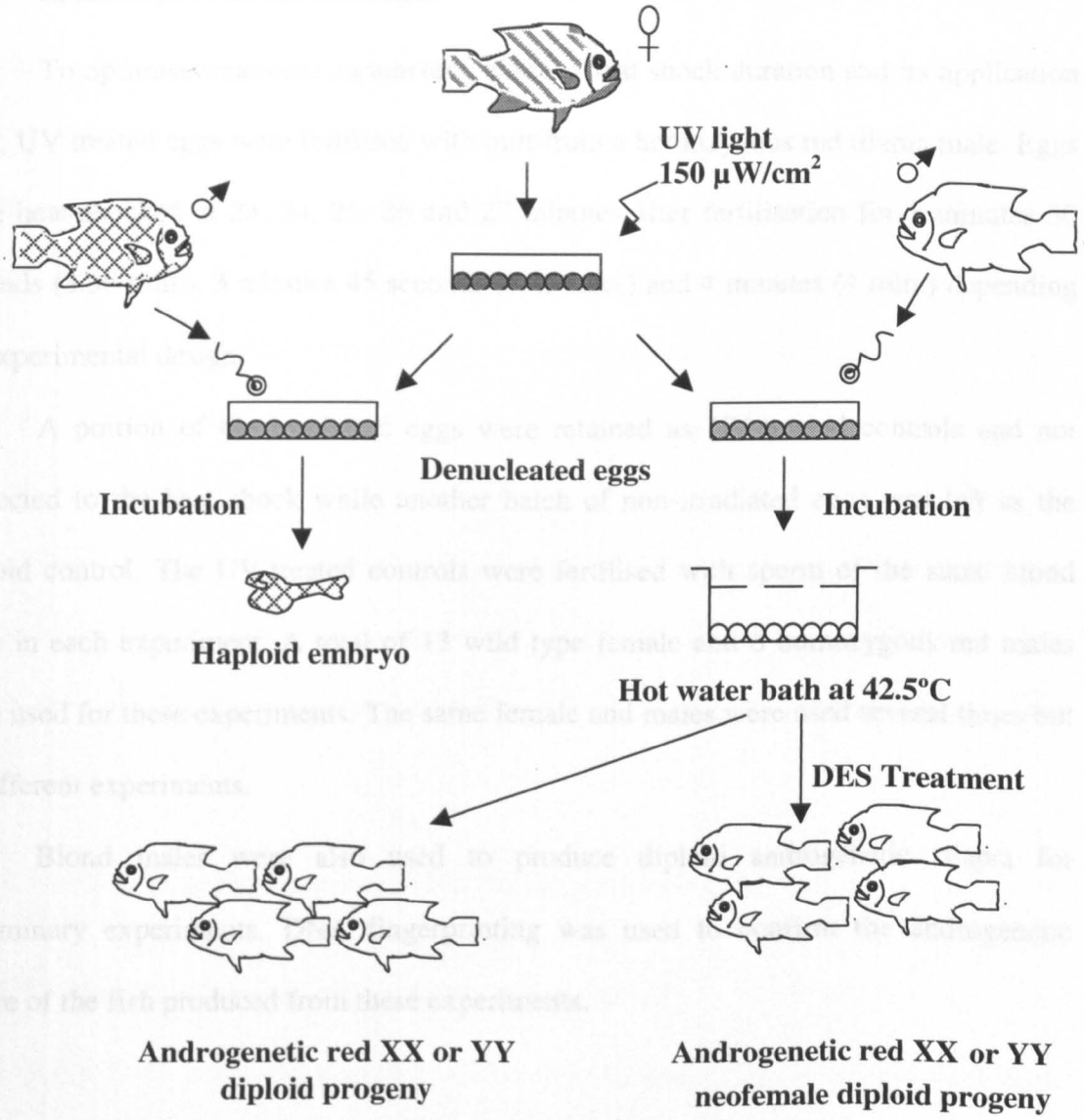


Figure 2.3. Schematic diagram of androgenesis in tilapia (*O. niloticus*) to produce YY male and YY neofemale homozygous red tilapia.

Wild type tilapia

Homozygous red tilapia

Blond tilapia

2.2.12.2. Experimental design for optimisation of heat shock duration time and minutes post fertilisation time

To optimise treatment parameters, namely heat shock duration and its application time, UV treated eggs were fertilised with milt from a homozygous red tilapia male. Eggs were heat-shocked at 23, 24, 25, 26 and 27 minutes after fertilisation for 3 minutes 30 seconds (3.30 min.), 3 minutes 45 seconds (3.45 min.) and 4 minutes (4 min.) depending on experimental design.

A portion of the irradiated eggs were retained as UV-treated controls and not subjected to the heat shock while another batch of non-irradiated eggs was left as the diploid control. The UV-treated controls were fertilised with sperm of the same blond male in each experiment. A total of 13 wild type female and 6 homozygous red males were used for these experiments. The same female and males were used several times but in different experiments.

Blond males were also used to produce diploid androgenetic tilapia for preliminary experiments. DNA fingerprinting was used to confirm the androgenetic nature of the fish produced from these experiments.

2.2.13. Statistical analyses

Since the egg quality of each spawn varied greatly within and between females, the survival of each treatment was always calculated relative to the survival of their corresponding diploid control group. When the survival rate of the control group was less than 30 %, that particular batch of eggs was not included (Myers *et al.*, 1995a). The data from the results of morula stages were transformed to arc-sine for statistical analyses and

normality was tested by Anderson-Darling Normality test and a test for homogeneity of variance applied (Sokal and Rohlf, 1987). Only the results of morula stages were tested by one-way ANOVA since they were normally distributed. The other non-parametric data for pigmentation, hatching and yolk sac resorption stages, which included many zero values, were transformed to square root and tested by the Kruskal-Wallis Test (Sokal and Rohlf, 1987; Gardiner, 1997). The results were presented as mean and standard error of mean (±SE). One-way ANOVA test was performed for specific female and male effect using female and male tag number as factors. All statistical analyses were performed by Minitab 9.2 software.

The sex ratios of androgenetic males and DES treated androgenetic females were analysed by a Chi-square test to see whether the sex ratio was statistically different from 1:1 sex ratio or from the respective sex ratios in the normal control for that group of fish.

2.2.14. Extraction of total genomic DNA for fingerprinting

2.2.14.1. Sample collection, preparation, and digestion

For extraction of total genomic DNA, blood samples (fresh or frozen) were used. Blood samples were collected from the caudal vein of fish using 21-23 g sterile needles and syringes containing approximately 1 volume of Cortland's saline for 2 volumes of blood sample. After mixing in the syringe, blood samples were transferred into autoclaved 1.5 ml microfuge tubes then centrifuged at 1400 g for 2 mins. The supernatant was removed and the pelleted blood cells were stored at –20° C. 10µl of fresh or thawed blood cells was added to a mixture of 435 µl of TEN buffer (Appendix 1.1) and 10µl of DNase-free RNase (10 $mg.ml^{-1}$) in a 1.5 ml sterile microcentrifuge tube and mixed

gently. 50 µl of 10 % (w/v) Sodium dodecyl sulfate (SDS) solution was then added and the whole mixed again. After 30-60 min. incubation at 37°C, 10µl of proteinase K ($10mg.ml^{-1}$) was added to each sample and they were then incubated overnight at 37°C in a water bath.

In order to extract DNA, 500 µl of buffered phenol was added to the digested sample, mixed by gently shaking and inverting the tubes 10-15 times. The emulsion was centrifuged at 13000 rpm for 10 min. in a microcentrifuge at room temperature. The aqueous supernatant was transferred to another sterile 1.5 ml microtube using a micropipette and large bore sterile pipette tip to avoid shearing of long stranded DNA. The phenol extraction was repeated until the aqueous phase became clear. The aqueous DNA solution was extracted twice with 500µl of chloroform / isoamyl alchol (24: 1 v: v) following the same procedure as above. The aqueous phase from the last extraction was transferred to a new sterile microfuge tube and 0.6 volumes of isopropanol were added to the tube which was then shaken vigorously to mix. At this stage the DNA pellet was generally visible: if not, the tube was kept at –20°C (Maniatis *et al.*, 1982) for 2 h or –70°C for 30 min. to ensure complete precipitation. The precipitate was then pelleted at 13000 rpm for 10 min. The supernatant was removed leaving the pellet untouched and then the pellet was washed twice with 70 % ethanol (approximately 30 min. between washes). The pellets were dried at room temperature and resuspended in 100 µl of TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA) for 1 h at 37°C in a water bath and then stored at 4°C until further use.

2.2.14.2. Quantitation of DNA

Two methods were used for measuring the concentration of DNA in the samples, spectrophotometry and gel electrophoresis.

2.2.14.2.1. Spectrophotometric determination of DNA

The concentration and purity of DNA was measured by comparing its optical density at 260 nm and 280 nm. The OD value at 260 nm allows estimation of the concentration of nucleic acids (DNA and RNA), while the reading at 280 nm determines the amount of protein in the same samples. At 260 nm an OD of 1 corresponds to approximately 50 $\mu g.ml^{-1}$ for double stranded DNA, 40 $\mu g.ml^{-1}$ for single stranded DNA and RNA and 20 $\mu g.ml^{-1}$ for single stranded oligonucleotides (Maniatis *et al.*, 1982) The ratio between O.D. 260 / O.D. 280 gives an estimate of the purity of the extracted nucleic acids. The O.D. ratio less than 1.8 indicates unextracted protein with the DNA, whereas a ratio higher than 2 shows the presence of RNA. Therefore, the ratio between the readings at 260 nm and 280 nm should be between 1.8-2. In the case of contamination with protein, Proteinase K was added to 200 $\mu g.ml^{-1}$ final concentration while in the presence of RNA, DNase free RNase A was added to a final concentration of 100 $\mu g.ml^{-1}$. After the samples were incubated at 37°C for 1 h, one phenol and one chloroform extraction were repeated as above.

Sample preparation for spectrophotometric reading, was done by mixing 5 µl of extracted DNA with 995 µl TE buffer, shaking vigorously and incubating at 37°C for 1 hour. The diluted sample was transferred to a semimicro-UV cuvette (BDH) and 1 ml TE

buffer was placed in another cuvette as a reagent blank. The amount of DNA was calculated as below;

[DNA] = O.D. 260 x Dilution factors x 50 = X ugml^{-1}

2.2.14.2.2. Gel electrophoresis

A small agorose gel of 80 ml (0.7 %) along with a known standard marker (λ *Hind III*) was also run to estimate the amount of DNA. 0.54 g of ultra pure agarose was mixed with 77 ml of 1 x TBE (Appendix 1.2.) in a 250 ml beaker, and then heated over a Bunsen burner with continuous stirring. 4 µl of ethidium bromide (10 mg.ml^{-1}) was added to the cooled gel (50-60°C) which was then poured into the gel mould (15 cm x 10 cm). 1 µg of marker DNA was loaded in one well and the samples into adjacent wells. The gel was run at 3-5 V.cm^{-1} for 2-2.5 h. A UV transilluminator was used for visualisation of the DNA in the gel which was then photographed with a Polaroid camera (Polaroid film type 665). The DNA concentration was determined by comparing the intensity of sample with a marker band with similar intensity. Calculation of the proportion of the selected band to the total marker weight gave an estimate of the DNA concentration of the samples.

2.2.15. Digestion of DNA with restriction endonuclease *Hinf I* for DNA fingerprinting

Hinf I (Stratagene) restriction enzyme was used to digest 5 µg of each DNA sample in a total volume of 100 µl. The required amount of sterile deionised distilled water was mixed with 10 µl of 10 x universal reaction buffer, 10 µl of spermidine

trihydrochoride (400mM), 1 µl of acetylated Bovine serum albumin (BSA) (10 $mg.ml^{-1}$) and 5 µg of the DNA sample in a sterile 1.5 ml microcentrifuge tube and then mixed thoroughly by shaking. The restriction enzyme of *Hinf* I (4 unit μg^{-1} of DNA) and one drop of mineral oil were added to the mixture. The reaction tube was pulse spun and incubated at 37°C for 14-16 h. The mineral oil on the top of the sample prevented evaporation during incubation. To speed up the working condition, a premix containing sufficient 10 x universal reaction buffer, spermidine trihydrochloride, acetylated BSA and partial amounts of sterile deionised water can be prepared as a premix solution. After aliquoting the premix solution (e.g 50 µl) to the individual tubes, the required amount of DNA and restriction enzyme were added and the total volume raised to 100µl by adding sterile water.

At the end of the incubation period, the reaction tube was pulse spun to collect any condensate and 200µl of TE buffer was added to each tube. 300 µl of buffer-equilibrated phenol was added and mixed to stop restriction enzyme activity. Reaction mixture tubes were spun at 10000 rpm for 5 min. After transferring the aqueous phase to a new sterile microcentrifuge tube, it was re-extracted with an equal volume of chloroform : isoamyl alcohol (24:1). The final aqueous solution was recovered by mixing with 1:50 volume of 5 M NaCl solution and precipiting the restricted DNA by adding and mixing of 2.5 volumes of 100 % ethanol and holding at –20°C for 2 h. Restricted DNA was recovered by centrifugation at 13000 rpm for 15 min. at room temperature to obtain the precipitated DNA at the bottom of the tube. The DNA pellet was washed once with 1 ml of 70 % ethanol and spun as above. The supernatant was removed by pipetting and the DNA pellet was dried *in vacuo*. 6µl of TE buffer was added to the dried pellet which was then incubated at 37°C for 1 h. and stored at 4°C until further use.

2.2.15.1. Agarose gel electrophoresis

The restricted DNA was run in 0.7 % agarose gel (200 ml) which was prepared by mixing 1.4 g of ultra pure agarose (Gibco BRL) with 200 ml of 1 x TBE buffer in a 500 ml Erlenmeyer flask and heating the mixture with a Bunsen burner with occasional swirling (to ensure even mixing) until no agarose particles could be seen. When the agarose cooled to about 50°C the melted agarose was poured into a gel mould (20 cm x 20 cm) in which a comb with 30 wells was placed. Any bubbles on the surface were removed before the gel set. While the gel was cooling and solidifying, restricted DNA samples were prepared for loading by mixing with 2 µl of 10 x tracking dye (0.1 % bromophenol blue, 40 % Ficoll) and pulse spun to concentrate the samples at the bottom of the tube. The gel was immersed in a maxi-gel (Pharmacia LKB) bath containing 2.1 litres of 1 x TBE buffer, covering the gel to a depth of about 1-2 mm. The comb was gently removed and any air bubbles trapped in the wells were removed. The samples including tracking dye and a suitable size marker (λ *Hind III*) were loaded into the wells slowly by using a 10 µl adjustable micropipette. The gel was run at 1.5 Vcm^{-1} for 14-16 h. until the bromophenol blue had migrated about three-fourths of the gel distance. After the gel run was completed, 0.5µg.ml^{-1} ethidium bromide was added into the gel bath and shaken gently for 15-20 min. The gel was washed with deionised H_2O for 10-15 min., placed on a long wavelength UV transilluminator (UVB), and visualised to ensure that complete digestion of the DNA had taken place. The migration distance of the marker DNA bands was recorded and the gel was photographed using a Polaroid camera. The gel was kept at 4°C.

2.2.15.2. Southern Blotting of restricted DNA

Southern transfer of restricted DNA to a non-charged membrane was carried out by means of alkaline vacuum blotting (Vacu Gene™, Pharmacia LKB). The blotting system uses a low vacuum pressure to transfer nucleic acids from an agarose gel to a transfer membrane. The blotting tray was prepared by washing with deionised water. A 20 x 20 cm non-charged nylon membrane (Hybond-N, Sartorius Ltd) was pre-wetted with deionised water and placed on the pre-wetted porous screen with the shiny side up by means of flattened forceps. A plastic mask with a window 5 mm smaller than the membrane was then placed to overlap the membrane on all sides by at least 2 mm. Any air bubbles beneath the membrane were removed. Four locking clamps tightened the top frame of the blotting unit.

The vacuum pump was checked by observing the disappearance the 10 ml of dionised water added on the membrane when the pump was on. The gel from the fridge was then placed onto the centre of the membrane starting with one of its edges and then gradually slid onto the membrane. The gel was readjusted if required by smoothing the gel using a gloved finger in a gentle stroking fashion. The gel and the mask have to overlap by at least 2 mm. When there were no gaps between the mask and gel, the pump was turned on and enough 0.2 N HCl solution immediately poured onto the centre of the gel to just cover it. Acid-alkaline treatment makes the gel stronger and minimises any collapse during transfer. When the bromophenol blue dye in the gel just turned yellow (20-25 min.), the HCl solution was pipetted out from the vacuum unit using a 5 ml automatic pipette. One litre of 0.4 M NaOH solution was immediately poured onto the middle of the gel and left for 65-70 min. This step was employed to produce single

stranded DNA that would be able to hybridise with the complementary DNA probe. While the pump was on, the NaOH solution was poured off and the pump was turned off. The well positions were marked with a blunt pencil and the gel was lifted off. The membrane was placed into a tray containing 500 ml 2 x Standard saline citrate (SSC) solution (Appendix 1.3.) and washed for 10 min. to neutralise the denaturation transfer solution and to remove any agorose particles stuck to the filter. The membrane was then air-dried at room temperature for 30 min. and finally placed between two sheets of clean 3 mm filter paper and incubated at 80°C. At the end of the incubation period, the membrane was stored at room temperature until further use.

2.2.15.3. Hybridisation of Southern blot membrane with non-Isotopic Chemiluminescent Enhanced (NICE™) Probe (33.15)

Four steps (pre-washing, pre-hybridisation, hybridisation and post-hybridisation washing) were employed to hybridise the NICE™ probe 33.15 (Cellmark Diagnostic) to transferred single stranded DNA fragments.

2.2.15.3.1. Pre-washing

The membrane was wetted by putting it in a clean tray containing 250 ml of 1 x SSC solution and placed into a hybridisation canister with the DNA side inwards. Air bubbles were squeezed out by rolling a pipette along the canister and 50 ml of pre-wash solution (0.1xSSC, 0.5 % SDS) prewarmed to 65°C was added. The canister containing the membrane and pre-wash solution was placed in a hybridiser (HB-1, TechNe) and left

for 1 h at 65°C. Any agarose and/or other particles stuck to the membrane which might cause unwanted background during hybridisation would be removed by this stage.

2.2.15.3.2. Pre-hybridisation

Following pre-washing the canister was removed from the hybridiser, the pre – wash solution poured off and replaced by 50 ml of prewarmed (50°C) pre-hybridisation buffer (990 $ml.l^{-1}$ of 0.5 M Na_2HPO_4, 10 $ml.l^{-1}$ of 10 % SDS). The canister was put back into the hybridiser and left for 20 min. at 50°C.

2.2.15.3.3. Hybridisation

20 ml hybridisation buffer (stock solution: 900 $ml.l^{-1}$ of pre-hybridisation buffer and 100 ml of 10 % w/v casein solution) were prepared and pre-warmed to 50°C in a sterile universal (BDH). Just before replacing the pre-hybridisation buffer, 5 µl of NICE™ probe 33.15 was added to the hybridisation buffer and mixed gently. The pre-hybridisation solution was discarded and replaced with hybridisation solution containing the probe. The hybridisation step was carried out for 20 min at 50°C.

2.2.15.3.4. Post-hybridisation washing

The filter was rinsed twice for 10 min. each at 50°C with 50 ml of wash solution 1 (160 $ml.l^{-1}$ of 0.5 M Na_2HPO_4, pH 7.2; 10 $ml.l^{-1}$ of 10 % SDS) pre-warmed to 50°C. Finally, the membrane was washed twice with 50 ml of wash solution 2 (13.8 $g.l^{-1}$ of

maleic acid, $C_4H_3O_4Na$; 8.7 $g.l^{-1}$ of NaCl, pH 7.2) for 5 min. at room temperature. The membrane was then removed from the canister and placed with the DNA side up on a clean glass plate. Approximately 3-4 ml of Lumi Phos™ 350 (Cellmark Diagnostics) was sprayed evenly over the membrane by means of a spray gun (BDH). The sprayed membrane was then sandwiched between two 21x21 cm acetate sheets and any excess Lumi Phos™ 350 squeezed out by rolling a pipette, avoiding contaminating the outer surface of the acetate sheets.

2.2.15.4. Autoradiography

The sandwiched membrane was trimmed with a sterile, sharp scalpel to fit the size of the 18x24 cm light proof Hypercassette. Each edge of the membrane was secured by using small pieces of sticky tape. The membrane was placed in an X-ray cassette and a sheet of autoradiography film (Hyperfilm MP™, Amersham) was then laid on it in a dark room. The right-top hand corner of the film was marked with a waterproof pen. The cassette was firmly closed and kept in an incubator at 30°C for at least 6 h. and then developed. Another sheet of film was then placed on the membrane in the dark room and kept in the incubator at 30°C for another 12 h. At the end of exposure time, the film was developed with continuous agitation in D19 developer (Kodak) until the bands were seen. The film was then removed from the developer and transferred to X-ray fixer (Kodak) for 1-2 min. The fixed film was washed in running water for 10-15 min. The autoradigraph was then air dried and kept at room temperature.

2.3. Results

2.3.1. Optimisation of UV treatment duration

The effect of UV exposure for 2, 4, 6, 8, 10 and 12 min on the percentage of morula, the percentage of pigmented and unpigmented embryo and the percentage of abnormal embryos in the presumptive haploid "blond" androgenetic Nile tilapia are presented in Table 2.4. and Fig. 2.4. All measurements are in relation to untreated controls.

At morula stage, all the levels of fertilisation of treatment groups and control were quite similar and there were no significant differences between them ($P>0.05$). As can be seen in Fig. 2.4 A, the fertilisation levels decreased with increased UV exposure time.

At pigmentation stage, the survival of averaged pigmented (38.29±1.18 %) and unpigmented embryos (39.56±0.97), were not significantly different in the control group indicating that the females used in these experiments were heterozygous for the blond locus. For 2 and 4 min UV duration time, pigmented embryos were observed at a survival rate of 5.51±1.93 % and 0.22±0.22 %, respectively. Although the highest survival of unpigmented embryos (46.03±13.6 %) was obtained for 2 min UV duration time, these treatments contained pigmented embryos showing only partial success with oocyte denucleation. 6 min UV exposure time with a survival rate of 18.53±5.3 % provided the best survival amongst the treatments giving only blond embryos. The survival rates of the treatment groups declined with increasing UV exposure times.

At hatching stage, there were no significant differences between normal developed pigmented and unpigmented embryos with a survival rate of 32.14±1.08 % and 35.48±2.10 %, respectively, in control group ($P>0.05$). In the 2 min treatment group, survival rates of

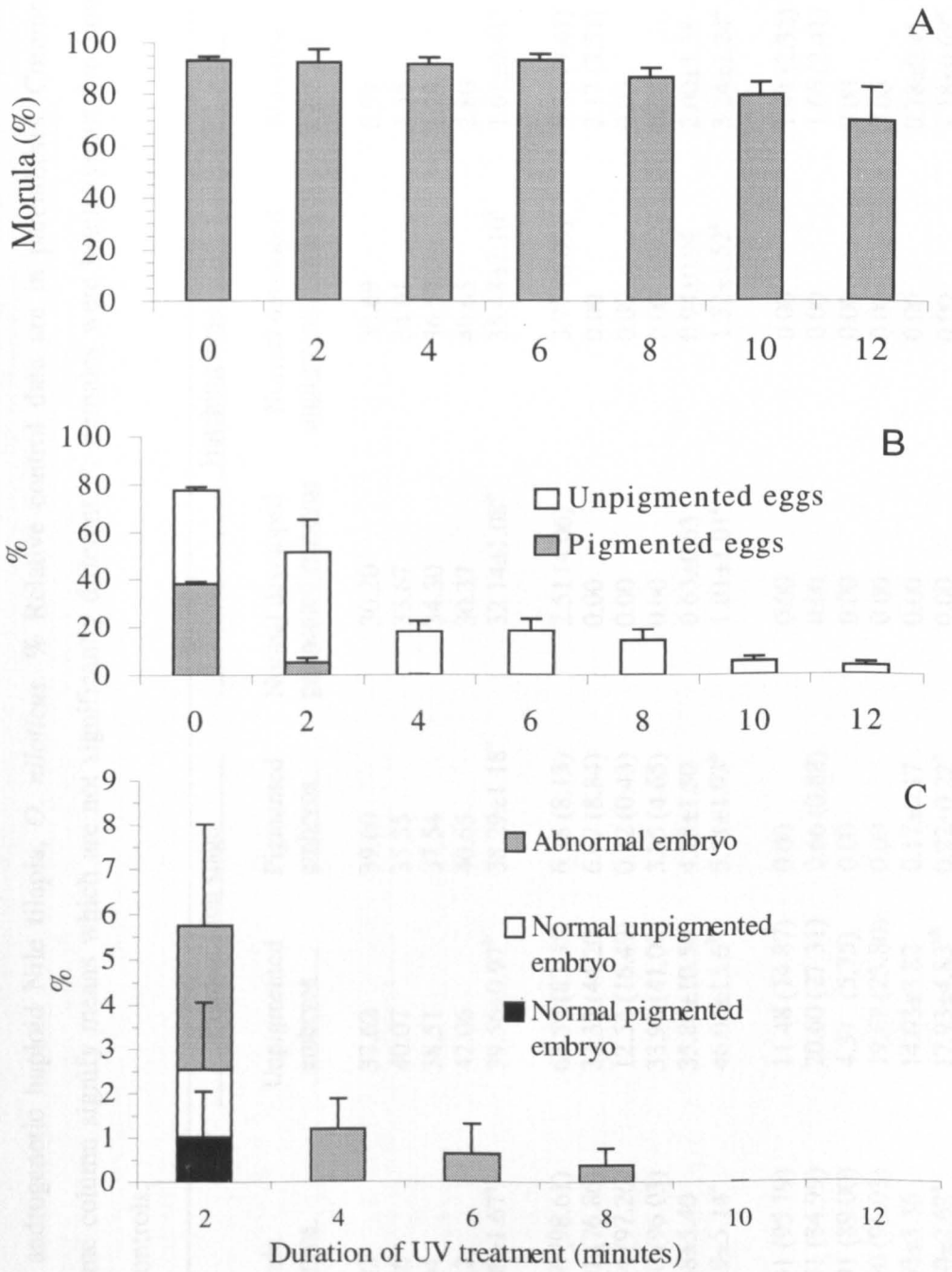


Figure 2.4. Percentage of embryos observed at morula (A), pigmentation (B) and hatching (C) of presumptive "blond" androgenetic haploid Nile tilapia, *O. niloticus*, (% relative to the diploid control) subjected to 150 μWcm^{-2} intensity.

Table 2.4. The effect of UV exposure (at 150 μWcm^{-2}) for 2, 4, 6, 8, 10 and 12 min on the morula, pigmentation and hatching of presumptive "blond" androgenetic haploid Nile tilapia, *O. niloticus*. % Relative control data are in parentheses. Common superscripts in the same column signify means which are not significantly different. *: Females were heterozygous for blond gene, R: Relative to controls.

			Pigmentation stage		Hatching stage		
UV dose (Minute)	Experiment no	Morula embryos	Unpigmented embryos	Pigmented embryos	Normal developed pigmented embryos	Normal developed unpigmented embryos	Abnormal embryos
0	1*	98.02	37.62	39.60	30.20	30.69	0.99
	2*	92.26	40.07	35.35	33.67	34.01	1.35
	3*	90.94	38.51	37.54	34.30	36.57	1.29
	4*	91.12	42.06	40.65	30.37	40.65	2.80
	Mean	93.08±1.67^{a}	39.56±0.97^{b}	38.29±1.18^{c}	32.14±1.08^{b}	35.48±2.10^{b}	1.61±0.41^{a}
2	1*	96.66 (98.61)	63.59 (82.35)	6.28 (8.13)	2.51 (4.06)	3.77 (6.09)	5.86 (9.47)
	2*	70.91 (76.86)	33.33 (44.20)	6.67 (8.84)	0.00	0.00	2.17 (3.51)
	3*	88.44 (97.26)	12.54 (16.49)	0.32 (0.43)	0.00	0.00	0.00
	4*	87.50 (96.03)	33.98 (41.08)	3.85 (4.65)	0.00	0.00	0.00
	Mean	85.88±5.40	35.86±10.50	4.28±1.50	0.63±0.63	0.94±0.94	2.00±1.38
	Mean (R)	92.19±5.14^{a}	46.03±13.6^{b}	5.51±1.93^{b}	1.01±1.01^{a}	1.52±1.52^{a}	3.24±2.24^{a}
4	1*	93.31 (95.19)	11.48 (14.87)	0.00	0.00	0.00	1.44 (2.32)
	2*	78.41 (84.99)	20.60 (27.31)	0.66 (0.88)	0.00	0.00	1.66 (2.41)
	3*	80.91 (89.00)	4.37 (5.75)	0.00	0.00	0.00	0.00
	4*	87.50 (96.03)	19.68 (23.80)	0.00	0.00	0.00	0.00
	Mean	85.03±3.36	14.03±3.82	0.17±0.17	0.00	0.00	0.78±0.45
	Mean (R)	91.30±2.62^{a}	17.93±4.83^{ab}	0.22±0.22^{a}	0.00	0.00	1.18±0.69^{a}

Table 2.2 continued

UV dose (Minute)	Experiment no	Morula embryos	Pigmentation stage: Unpigmented embryos	Pigmentation stage: Pigmented embryos	Hatching stage: Normal developed pigmented embryos	Hatching stage: Normal developed unpigmented embryos	Hatching stage: Abnormal embryos
6	1*	92.55 (94.42)	14.36 (18.60)	0.00	0.00	0.00	0.00
	2*	82.74 (89.68)	23.02 (30.52)	0.00	0.00	0.00	1.80 (2.61)
	3*	82.24 (90.43)	3.62 (4.76)	0.00	0.00	0.00	0.00
	4*	89.73 (98.48)	16.73 (20.23)	0.00	0.00	0.00	0.00
	Mean	86.82±2.56	14.43±4.04	0.00	0.00	0.00	0.45±0.45
	Mean (R)	93.25±2.02[a]	18.53±5.3[ab]	0.00	0.00	0.00	0.65±0.65[a]
8	1*	81.10 (82.74)	7.20 (9.32)	0.00	0.00	0.00	0.00
	2*	72.32 (78.39)	19.72 (26.15)	0.00	0.00	0.00	1.04 (1.50)
	3*	79.83 (87.78)	4.04 (5.31)	0.00	0.00	0.00	0.00
	4*	87.96 (96.53)	14.38 (17.39)	0.00	0.00	0.00	0.00
	Mean	80.30±3.20	11.34±3.53	0.00	0.00	0.00	0.26±0.26
	Mean (R)	86.36±3.89[a]	14.54±4.62[a]	0.00	0.00	0.00	0.38±0.38[a]
10	1*	68.96 (70.35)	4.51 (5.84)	0.00	0.00	0.00	0.00
	2*	79.33 (85.98)	3.39 (4.49)	0.00	0.00	0.00	0.00
	3*	65.58 (72.11)	1.31 (1.72)	0.00	0.00	0.00	0.00
	4*	81.93 (89.91)	9.35 (11.30)	0.00	0.00	0.00	0.00
	Mean	76.45±5.93	4.64±1.70	0.00	0.00	0.00	0.00
	Mean (R)	79.59±4.91[a]	5.83±2.01[a]	0.00	0.00	0.00	0.00
12	1*	68.12 (69.50)	3.18 (4.12)	0.00	0.00	0.00	0.00
	2*	74.83 (81.11)	5.86 (7.77)	0.00	0.00	0.00	0.00
	3*	31.10 (34.20)	0.79 (1.04)	0.00	0.00	0.00	0.00
	4*	86.40 (94.82)	2.94 (3.56)	0.00	0.00	0.00	0.00
	Mean	65.11±11.96	3.19±1.04	0.00	0.00	0.00	0.00
	Mean (R)	69.91±12.98[a]	4.12±1.38[a]	0.00	0.00	0.00	0.00

1.01±1.01 %, 1.52±1.52 % and 3.24±2.24% were observed in normally developed pigmented and unpigmented embryos and abnormal embryos, respectively. Only abnormal embryos were produced in 4, 6 and 8 min treatments while 10 and 12 min UV duration time did not result in any hatched embryos. There were no significant differences between treatments in terms of abnormality (P>0.05). None of the UV treated embryos, including pigmented embryos, in the 4, 6, 8, 10 and 12 min treatments survived more than a few days post-hatching.

Analysis of some of the embryos by karyological examination (Fig. 3.1) showed a typical single set of chromosomes (n=22: Majumdar and McAndrew, 1983b).

2.3.2. Optimisation of post-fertilisation, heat shock start and duration time using eggs subjected to 6 minutes UV exposure

The results of optimisation of UV exposure time showed that 6 min treatment is the most effective in inducing oocyte denucleation. Therefore optimisation of heat shock duration time (3. 30 min, 3. 45 min and 4 min) and application time (25 and 27 m.a.f.) were tested by applying 6 min UV exposure time to eggs to produce diploid androgenetic tilapia. Mean (±SE), minimum and maximum survival rates (relative to controls) to pigmentation and hatching stages of presumptive androgenetic red tilapia subjected to 6 min UV duration time and various thermal shocks applied various times after-fertilisation are depicted in Table 2.5. and Fig. 2.5. No significant differences were found between treatment parameters of 25 and 27 m.a.f and 3. 30 min, 3. 45 min and 4 min heat shock duration time at pigmentation stage (P>0.05). The highest survival of 2.14±2.14% was obtained in the group of 3.45 min heat shock duration time applied 25 m.a.f. at pigmentation. Only the group of 25

Table 2.5. Minimum, maximum and mean (±SE) survival (%relative to the diploid controls) to pigmentation and hatching stages of presumptive diploid androgenetic Nile tilapia, *O. niloticus*, subjected to 6 minutes prefertilisation UV duration time and thermal shock duration at 42.5°C at various times after-fertilisation. Common superscripts in the same column indicate means which are not significantly different. n: number of replicates.

Minutes after-fertilisation	Heat shock duration time	n		Pigmentation	Hatching
25	3 min 30 sec	4	Mean	1.23±1.23[a]	0.61±0.61
			Min.	0.00	0.00
			Max.	4.92	2.43
25	3 min 45 sec	4	Mean	2.14±2.14[a]	0.00
			Min.	0.00	0.00
			Max.	8.56	0.00
25	4 min	4	Mean	0.85±0.85[a]	0.00
			Min.	0.00	0.00
			Max.	3.41	0.00
27	3 min 30 sec	4	Mean	0.52±0.52[a]	0.00
			Min.	0.00	0.00
			Max.	2.10	0.00
27	3 min 45 sec	4	Mean	1.05±1.05[a]	0.00
			Min.	0.00	0.00
			Max.	4.20	0.00
27	4 min	4	Mean	0.00	0.00
			Min.	0.00	0.00
			Max.	0.00	0.00

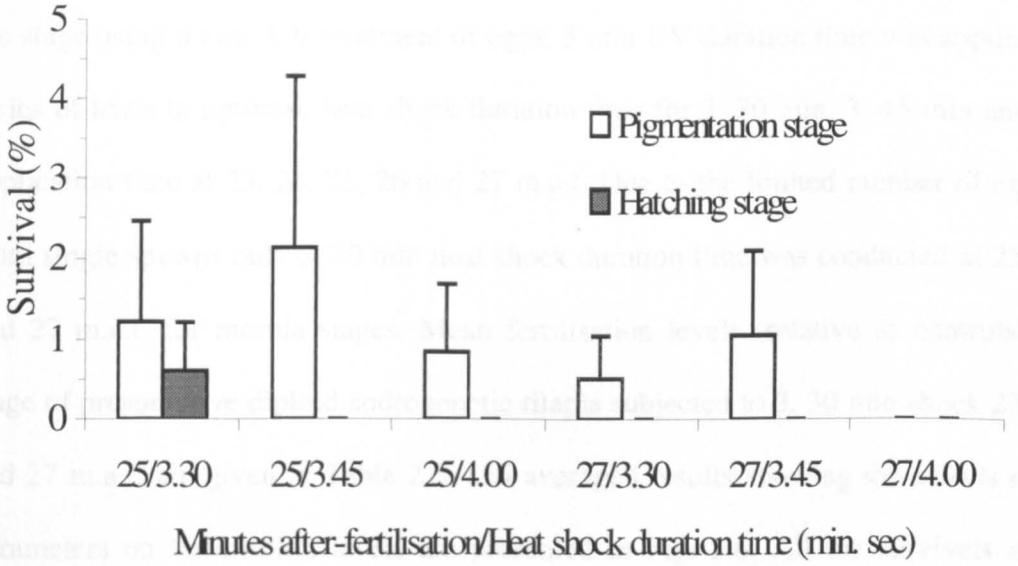


Figure 2.5. Average survivals (% relative to the diploid controls) of presumptive diploid androgenetic Nile tilapia, *O. niloticus*, embryos to pigmentation and hatching stage subjected to 6 minutes UV irradiation time and various heat shock times after fertilisation and durations.

m.a.f. at pigmentation. Only the group of 25 m.a.f heat shocked for 3. 30 min hatched with a survival rate of 0.61±0.61 %. No embryos reached yolk sac resorption stage.

2.3.3. Optimisation of post-fertilisation heat shock start and duration time, using eggs subjected to 5 minutes UV exposure

As a result of the lack of production of viable diploid androgenetic red tilapia to yolk sac stage using 6 min. UV treatment of eggs, 5 min UV duration time was applied in another series of trials to optimise heat shock duration time for 3. 30 min, 3. 45 min and 4 min and application time at 23, 24, 25, 26 and 27 m.a.f. Due to the limited number of eggs obtained from single spawns only 3. 30 min heat shock duration time was conducted at 23, 24, 25, 26 and 27 m.a.f. for morula stages. Mean fertilisation levels (relative to controls) to morula stage of presumptive diploid androgenetic tilapia subjected to 3. 30 min shock 23, 24, 25, 26 and 27 m.a.f. are given in Table 2.6. and averaged results showing the effects of treatment parameters on fertilisation levels are presented in Fig. 2.6. All the survivals of treatment groups, their respective controls and haploid controls were almost equal and not significantly different ($P>0.05$). The highest fertilisation level of 96.62±2.54 % was obtained in the haploid control group while the lowest fertilisation level of 84.14±9.41 % was found when the denucleated eggs were subjected to a 3. 30 min heat shock at 24 m.a.f. A maximum fertilisation level of 99.64 to 100 % was observed in some experiments in all treatment groups.

Before giving the results of optimisation treatments for pigmentation, hatching and yolk sac stages, it is convenient to begin by first explaining the experimental design to emphasise the reason for the different heat shock durations applied at different times. Because of the limited number of eggs not all treatment parameters could be tested on the same batch of eggs, only a few treatment parameters could be tested per experiments on UV treated eggs. The most effective heat shock duration and application times to induce diploid

Table 2.6. Mean (±SE) fertilisation levels (% relative to the diploid controls) to morula stage of presumptive diploid androgenetic Nile tilapia, *O. niloticus*, subjected to 5 minutes UV exposure time and thermal shock duration of 3 min 30 sec at 42.5°C at various times after-fertilisation. Common superscripts in the same column indicate means which are not significantly different. n: number of replicates.

Minutes after-fertilisation	n		Morula stage
Control	4	Mean	94.22±1.82[a]
		Min.	89.89
		Max.	98.29
Haploid Control	4	Mean	96.62±2.54[a]
		Min.	90.54
		Max.	100
23	4	Mean	88.28±6.70[a]
		Min.	76.04
		Max.	100
24	4	Mean	84.14±9.41[a]
		Min.	63.02
		Max.	100
25	4	Mean	92.84±4.73[a]
		Min.	79.63
		Max.	100
26	4	Mean	90.73±5.40[a]
		Min.	78.48
		Max.	100
27	4	Mean	85.58±5.36[a]
		Min.	73.75
		Max.	99.64

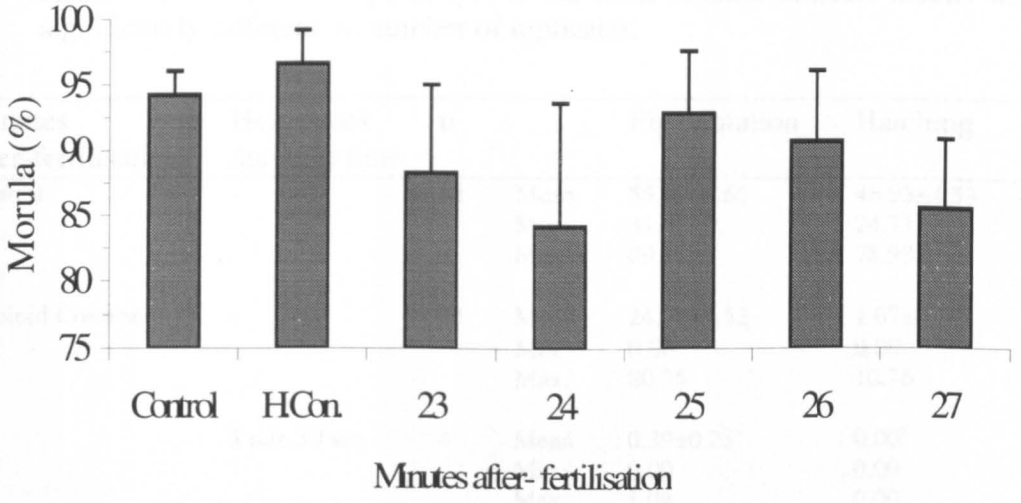


Figure 2.6. Average morula (% relative to the diploid controls) of presumptive androgenetic Nile tilapia, *O. niloticus*, embryos subjected to 5 minutes UV irradiation time and 3 min 30 sec heat shock duration time at various times after-fertilisation time (H. Con: haploid control).

androgenetic tilapia were reported to be between 3-4 min at 42.5°C and 25-27.5 min by Myers *et al.* (1995a). Therefore, most of the experiments were conducted in these windows. The mean (±SE), minimum and maximum survival rates (relative to controls) to pigmentation, hatching and yolk sac resorption stages of presumptive red androgenetic tilapia subjected to various heat shock duration times at various times after fertilisation are given in Table 2.7. and averaged survivals are presented graphically in Fig. 2.7. At pigmentation stage, peak survivals of 10.69±2.24 %, 11.14±4.54 % and 7.91±1.90 % were obtained in the treatment of 25, 26 and 27 m.a.f. for 3. 30 min heat shock duration time, respectively, and they were significantly differed from the other treatment groups ($P<0.05$).

Table 2.7. Mean (±SE) survival (%relative to the diploid controls) to pigmentation, hatching and yolk sac stages of presumptive androgenetic Nile tilapia, *O. niloticus*, subjected to 5 minutes UV irradiation and various thermal shock duration after various times after-fertilisation. Common superscripts in the same column indicate means which are not significantly different. n: number of replicates.

Minutes after-fertilisation	Heat shock duration time	n		Pigmentation	Hatching	Yolk sac resorption
Control	-	32	Mean	55.47±2.65	46.93±4.53	42.02±4.53
			Min.	31.45	24.71	24.47
			Max.	89.97	78.93	60.47
Haploid Control	-	32	Mean	24.58±3.52	1.67±0.58	0.00
			Min.	0.00	0.00	0.00
			Max.	80.35	10.76	0.00
23	3 min 30 sec	4	Mean	0.39±0.25[a]	0.00[a]	0.00[a]
			Min.	0.00	0.00	0.00
			Max.	1.04	0.00	0.00
24	3 min 30 sec	4	Mean	0.69±0.51[a]	0.00[a]	0.00[a]
			Min.	0.00	0.00	0.00
			Max.	2.17	0.00	0.00
25	3 min 30 sec	32	Mean	10.69±2.24[b]	2.03±0.60[b]	0.07±0.07[a]
			Min.	0.00	0.00	0.00
			Max.	50.63	11.71	2.34
25	3 min 45 sec	13	Mean	2.53±1.48[a]	0.50±0.50[a]	0.00[a]
			Min.	0.00	0.00	0.00
			Max.	18.96	6.56	0.00
25	4 min	9	Mean	0.80±0.43[a]	0.10±0.10[a]	0.00[a]
			Min.	0.00	0.00	0.00
			Max.	3.82	0.89	0.00
26	3 min 30 sec	14	Mean	11.14±4.54[b]	1.17±0.73[b]	0.03±0.03[a]
			Min.	0.00	0.00	0.00
			Max.	58.27	10.36	0.35
27	3 min 30 sec	28	Mean	7.91±1.90b[b]	1.21±0.53[b]	0.03±0.03[a]
			Min.	0.00	0.00	0.00
			Max.	32.88	11.86	0.72
27	3 min 45 sec	9	Mean	0.81±0.50[a]	0.00[a]	0.00[a]
			Min.	0.00	0.00	0.00
			Max.	4.17	0.00	0.00
27	4 min	9	Mean	1.40±0.84[a]	0.18±0.12[a]	0.00[a]
			Min.	0.00	0.00	0.00
			Max.	7.77	2.34	0.00

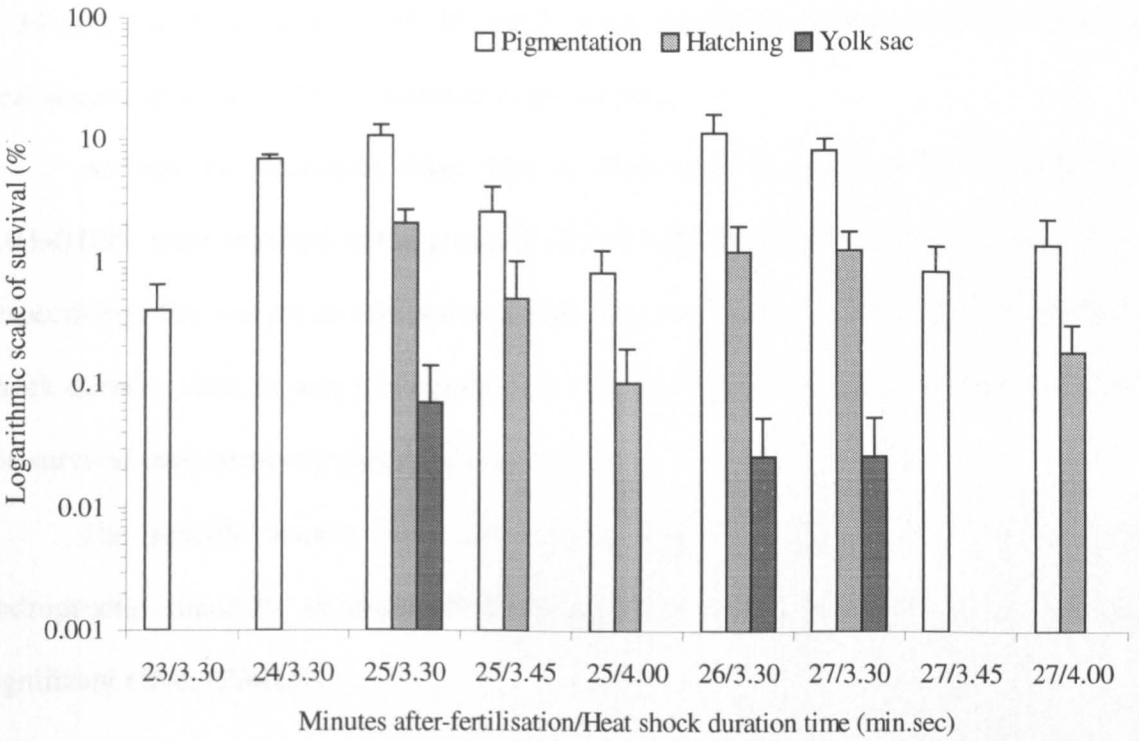


Figure 2.7. Average survivals (%relative to the diploid controls) of presumptive diploid androgenetic Nile tilapia, *O. niloticus*, embryos to pigmentation, hatching and yolk sac stage subjected to 5 minutes UV irradiation and various heat shock durations at various times after-fertilisation.

The survivals to pigmentation stage varied from zero for all treatment parameters to a maximum of 58.27% for 26 m.a.f. for 3. 30 min heat shock duration time. Although high survival rates were obtained at the pigmentation stage, survival rates decreased sharply by hatching stage.

There were significant differences between the treatment of 25, 26 and 27 m.a.f for 3. 30 min heat shock duration time and the other treatment groups ($P<0.05$) at hatching stages. The highest survival of 2.03±0.60 % was observed in the group of 25 m.a.f heat shocked for

3. 30 min. Late heat shocks of 25, 26 and 27 m.a.f. resulted in increased survival rates while heat shocks of 23 and 24 m.a.f resulted in no survival.

At yolk sac resorption stage, low survival rates of 0.07±0.07%, 0.03±0.03% and 0.03±0.03% were obtained in the group of 25, 26 and 27 m.a.f. heat shocked for 3. 30 min, respectively. The maximum survival of 2.34% was observed for 25 m.a.f. and 3. 30 min heat shock duration time in only one experiment. There were no significant differences between the survival rates of any treatment group ($P>0.05$).

The specific female used did have a significant effect on the percentage of androgenetic tilapia for all stages ($P<0.05$) but the male used in each cross did not have a significant effect ($P>0.05$).

2.3.4. Effect of egg quality

The survival rates of haploid controls subjected to 5 min UV-irradiation (Fig.2.8 A) and a diploid treatment group subjected to heat shock at 42.5°C for 3. 30 min at 25 m.a.f (Fig.2.8 B) were plotted against their respective controls and the trend lines were compared to examine the effect of egg quality on the survival rates of androgenetic haploid and diploid tilapia. The regression analyses showed that there was no significant relationship between control and haploid control survivals (correlation coefficient (r)=0.335, $P>0.05$) and between control and treatment group survivals (r=0.294, $P>0.05$).

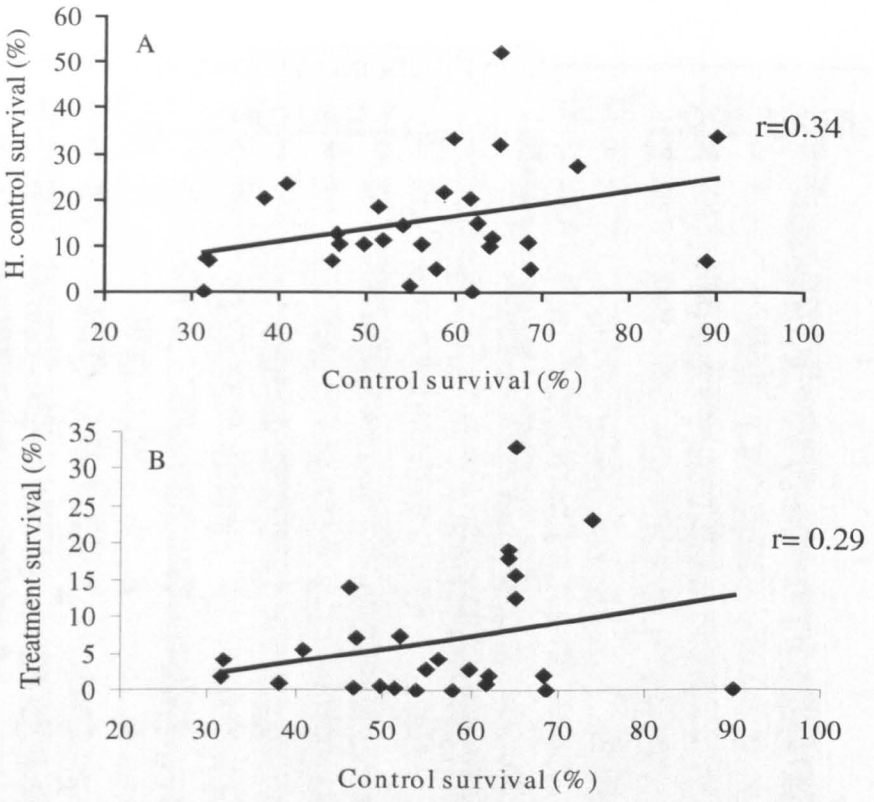


Fig.2.8. Plotted survival rates and fitted trend lines of androgenetic haploid controls subjected to 5 min UV irradiation (A) and a diploid androgenetic treatment group subjected to a heat shock at 42.5°C for 3 min 30 sec at 25 m.a.f. (B) against their respective diploid controls. (H: Haploid).

2.3.5. Verification of all-paternal inheritance by multilocus DNA fingerprinting

The multilocus DNA fingerprinting generated by using Jeffrey's 33.15 probe confirmed success in the production of diploid androgenetic tilapia. Fig. 2.9 showed that all

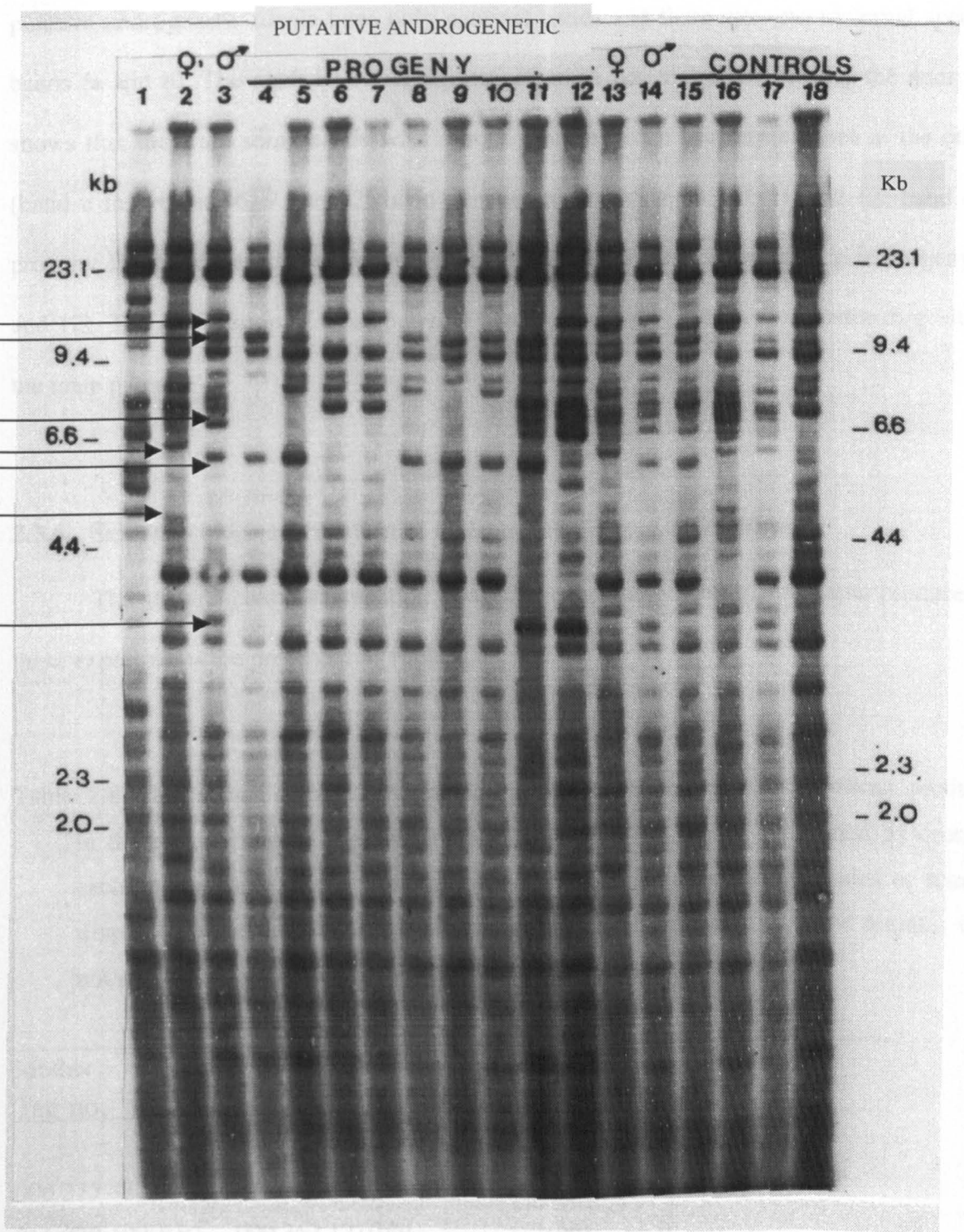


Figure 2.9. Scanned image of DNA fingerprinting of androgenetic tilapia. Lane 1: unrelated control, Lane 2 and 13: mother, Lane 3 and 14: father, Lane 4-12: androgenetic offspring, Lane 15 and 18: control. For information on bands labelled a-g see text.

putative androgenetic tilapia have only paternal bands and there were no maternal specific bands (a and b). The analysis of segregation of some paternal bands among the androgen shows that there are some bands which are present in some progeny but not in the others (band c in progeny 6, 7 and 12; band d in progeny 4, 5, 8, 9, 10, 11 and 12; band e in progeny 11 and 12; band f in progeny 4, 5, 6, 8, 9, 10, 11 and 12 and band g in progeny 11 and 12). The segregation of these paternal bands presumably results from heterozygosity in the male parent.

2.3.6. Sex and colour segregation of androgenetic tilapia, *O. niloticus*

The sex and colour segregation of all androgenetic red and blond tilapia produced in these experiments are presented in Table 2.8.

Table. 2.8. The sex and colour segregation of all androgenetic tilapia, *O. niloticus*, produced in these experiments. The progeny testing for coloration was performed by crossing each androgenetic tilapia and their respective parents to wild type males or females tilapia. (BB and rr: Wild type; bb: Blond; RR: Red). Two DES-treated females were not included since their genotype was unclassified (see Table 2.10.).

Mother	Father	Androgenetic offspring	
Tag no.	Tag no.	Male	Female
006 275 872 (BB)	000 362 121(bb)	11(bb)	16(bb)
011 020 109(BB)	000 362 121(bb)	1(bb)	0
088 849 001(rr)	011 007 120(RR)	2(RR)	1(RR)
014 798 273(rr)	014 300 201(RR)	1(RR)	1(RR)
015 299 071(rr)	013 555 839(RR)	0	1(RR)
	Total	15	19

A total of 29 blond and 7 red androgenetic tilapia were produced and assessed at the age of 4 months during these experiments. The pooled sex ratio of 15 male: 19 female from all androgenetic tilapia (excluding unclassified DES-treated androgenetic females of 015 314 792 blond and 015 314 792 red tilapia, see Table. 2.10.) were not significantly different from 1: 1 expected ratio ($P>0.05$). Progeny testing for red or blond genotypes was performed by crossing each androgenetic male, female and their respective parents to wild type (rr or BB, respectively) male and female tilapia. The progeny of blond androgenetic tilapia were all wild type while the progeny of red androgenetic tilapia were all red but with heavy black blotching as expected.

Table 2.9. shows the result of progeny testing of total 14 blond and red androgenetic male crossed to ordinary wild type female (XX) tilapia. With the exception of one androgenetic male (013 620 833) tilapia which showed 84% male progeny, all of the other androgenetic male tilapia produced 100% male progeny. Highly significant differences were found in the sex ratios of all androgenetic males tilapia from expected 1 : 1 ratio indicating that all androgenetic males had the YY genotype. Their respective controls did not show significant differences from 1 : 1 ratio ($P>0.05$).

Feminisation of red and blond androgenetic tilapia to produce YYRR and YYbb neofemales was not successful. A total of 30 blond and 3 red androgenetic tilapia were treated with DES but the survival rate was very low during and after the feminisation period. At the age of 4 months, only 2 blond and 2 red DES-treated androgenetic tilapia survived. The result of progeny testing of these fish in crosses to a XX neomale is presented in Table 2.10. Two of the DES-treated androgenetic tilapia did not produce any viable eggs although

Table 2.9. Progeny test results of androgenetic red and blond males of *O. niloticus* and their respective controls. Androgenetic males (YY) were denoted as those producing progeny sex ratio significantly different from a 1:1 ratio. Same letters in the same column indicates that results derived from the same single control cross. (M: Male, F: Female, RR: Red, bb: Blond, $^{***}P<0.001$)

Androgenetic male	Number of progeny				Respective control				Proposed genotype
(Tag no)	M	F	%M	$\chi 2$	M	F	%M	$\chi 2$	
010 317 057	20	0	100.0	20.00^{***}	10	15	60.0	1.00	YY(bb)
013 354 595	20	0	100.0	20.00^{***}	17a	16a	51.5a	0.03a	YY(bb)
014 524 616	18	0	100.0	18.00^{***}	a	a	a	a	YY(bb)
013 556 535	24	0	100.0	24.00^{***}	a	a	a	a	YY(bb)
010 554 342	26	0	100.0	26.00^{***}	a	a	a	a	YY(bb)
014 383 330	14	0	100.0	14.00^{***}	17b	21b	44.7b	0.42b	YY(bb)
010 893 049	22	0	100.0	22.00^{***}	b	b	b	b	YY(bb)
000 360 821	14	0	100.0	14.00^{***}	26c	24c	52.0c	0.08c	YY(bb)
012 570 379	16	0	100.0	16.00^{***}	c	c	c	c	YY(bb)
015 286 567	16	0	100.0	16.00^{***}	c	c	c	c	YY(bb)
013 378 126	24	0	100.0	24.00^{***}	c	c	c	c	YY(bb)
013 630 048	30	0	100.0	30.00^{***}	c	c	c	c	YY(RR)
013 620 833	42	8	84.0	23.10^{***}	c	c	c	c	YY(RR)
013 296 291	21	0	100.0	21.00^{***}	18	10	64.3	2.29	YY(RR)

Table 2.10. Progeny test results of sex reversed androgenetic red and blond females of *O. niloticus* and their respective control. Androgenetic females were crossed to an XX neomale and sex ratios were compared to a 1:1 expected ratio. Androgenetic females (XX) were denoted as those producing sex ratios significantly different from a 1:1 ratio. (M: Male, F: Female, RR: Red, bb: Blond, ***P<0.001; †: No viable eggs were obtained)

DES-treated androgenetic females	Number of progeny				Proposed genotype
(Tag no)	M	F	%F	$\chi 2$	
014 312 864	0	15	100.0	15.00^{***}	XX(bb)
015 314 792 †	-	-	-	-	?
000 610 589	0	18	100.0	18.00^{***}	XX(RR)
015 314 792 †	-	-	-	-	?

they had a female phenotype. The other 2 DES-treated androgenetic tilapia were designated as normal females (XX) as these fish yielded all female progeny, significantly different from 1 : 1 ratio (P<0.001). The XX neomale used in these experiments was previously tested by crossing to an ordinary female (XX), which produced 90% female progeny, significantly different from a 1 : 1 ratio showing that this male had an XX genotype.

2.4. Discussion

The present studies indicate that UV irradiation was successfully inactivating the nuclear DNA in Nile tilapia eggs. The yields of viable denucleated eggs to pigmentation stage varied between 0 to 85.35 % (relative to the controls) with a mean of 24.58±3.52 and 4.76 to 30.52 % (relative to the controls) with a mean of 18.53±5.3 % for 5 and 6 min UV duration time total dose of 450-540 Jm^{-2}, respectively. The yield is comparable with that of 22.9±1.6 % in Nile tilapia (Myers *et al.*, 1995a), 22% in loach (Arai *et al.*, 1995) and 22.5±2.8 % in muskellungen, *Esox masquinong*y (Lin and Dabrowski, 1998). In common carp, an optimal dose of 2500Jm^{-2} produced 53.9 % surviving haploids at hatching as well as a few biparental diploids (Bongers *et al.*, 1994). Bongers *et al.* (1995) were able to produce higher numbers of androgenetic haploids (81% to hatching, relative to control) in African catfish using an optimum UV dose of 1250 Jm^{-2}. Arai *et al.* (1995) successfully produced 22 % hatched androgenetic haploids in loach with a dose of 750 Jm^{-2}. The yield of haploid androgenetic muskellunge was 22.5±2.8 % with optimal UV irradiation doses of 620-1320 Jm^{-2} (Lin and Dabrowski, 1998). Marengoni and Onoue (1998) obtained survival rates of 57.6% & 55.8% and 57.1% & 56.0% (relative to controls) in androgenetic haploid *O. aureus* and *O. niloticus*, respectively, at total UV dose of 594 and 693 Jm^{-2}, respectively.

By using the recessive "blond" skin pigmentation character in spermatozoa, it was possible to assess whether oocyte denucleation was successful. All haploid embryos showed non-pigmentation under optimal UV irradiation of 5-8 minutes whereas 2 min and 4 min UV irradiation produced some pigmented embryos. The blond colour variant was also used successfully by Myers *et al.* (1995a) in the production of androgenetic haploid tilapia and they also observed some pigmented embryos in the same UV irradiation treatments as the

present study. Pigmented embryos were observed in the 2 and 4 min UV treatments and blond embryos showed aberrant development. Therefore, to ensure that host eggs are totally denucleated, the UV treatment should be at least 5 min to 8 min at 150 μWcm^{-2} or a total dose of between 450 Jm^{-2} and 720 Jm^{-2}. Myers *et al.* (1995a) reported that variable sensitivity to UV irradiation from species to species could be explained by differences in the thickness, composition and optical qualities of egg chorion, egg size and shape, and the relative position of the female pronucleus.

After establishing the optimal UV irradiation time (5 min at 150 μwcm^{-2} or 450 Jm^{-2}), diploid androgenetic red tilapia were obtained using a heat shock at 42.5°C for 3.30 min duration to inhibit the first mitotic division. The results of the present study demonstrate that androgenetic diploids can be produced in red Nile tilapia, although the percentage was extremely low. The survival rate reduced as development progressed (92.84±4.73 % to morula, 10.69±2.24 % to pigmentation, 2.03±0.6 % to hatching and 0.07±0.07 % to yolk sac resorption stage (relative to diploid controls) under optimal treatment parameters. Such poor viability was not surprising for androgenetic diploids as it was similar to that of mitotic gynogenetic diploids and tetraploid fish obtained by suppression of the first cleavage by physical shocks. Summary of survival rates of androgenesis and mitotic gynogenesis research is presented in Table 2.2 (page 41).

The reason for low survivals during the production of androgenetic diploid fish can be attributed to several factors. Firstly, radiation can induce structural chromosome aberrations which increase the dispersion of cellular DNA in proliferating cell populations (George *et al.*, 1991). The proportion of cells containing damaged chromosomes increases as embryos grow. Most of the chromosome aberrations arise directly after irradiation and may

be transmitted from cell to cell during the subsequent cleavage. Such aberrations exhibit a characteristic shape resulting from the breakdown of chromosome bridges, and have become known as "bridge fragments" (Pankova, 1965: cited in Egami and Ijiri, 1979). Radiation-induced chromosome damage has been suggested as the cause of an increase in intercellular variations in DNA content (George *et al.*, 1991). The effects of UV irradiation on eggs has been discussed by various authors. Firstly, it was suggested that the inactivation of maternal genomes and partial fragmentation of chromosomes did not prevent them from being involved in mitotic division (Lin and Dabrowski, 1998). They reported chromosomal fragments in some cells of abnormal larvae irradiated at 660 Jm^{-2} although the nuclear DNA content of haploids from groups irradiated at more than 660 Jm^{-2} did not differ from half the DNA content of control fish. DNA fragments, probably of maternal chromosome residues, were found in haploid androgenetic loach after UV inactivation of eggs (Arai *et al.*, 1992). Gillespie and Armstrong (1980) and Bongers *et al.* (1994) reported biparental diploids at the optimal UV dose to inactivate the female genome. This suggested that genetic contributions of the female may not be fully eliminated and possible maternal nuclear DNA fragments may be involved up to mitotic division (Carter *et al.*, 1991). Bongers *et al.* (1995) suggested that UV irradiation might damage the maternal RNA in eggs, which is essential for the development up to the blastula stage, and thus affect the differentiation process of embryonic development by altering cell fates and lineages. On the other hand, Myers *et al.* (1995a) reported that egg mtDNA in tilapia, analysed with ultraviolet endonuclease was not affected by doses of UV irradiation high enough to denucleate the eggs and suggested that UV irradiation had relatively little impact on the eggs beyond nuclear inactivation. Higher survival rates for androgenetic diploids produced using spermatozoa of artificial tetraploids

spermatozoa of artificial tetraploids have been reported in rainbow trout (Thorgaard *et al.*, 1990). They suggested that the radiation treatments to inactivate the maternal DNA in androgenesis are not an overwhelming obstacle to good survival. Parsons and Thorgaard (1984) found that androgenetic haploid and gynogenetic haploid rainbow trout showed similar survival rates, also suggesting that egg irradiation alone may not be responsible for excessive mortality. In the present study, in some experiments high survival to pigmentation stage (80.35 % for 5 min UV duration time and 30.52 % for 6 min UV duration time) were observed and the results agreed with those of Myers *et al.* (1995a), Thorgaard *et al.* (1990) and Parsons and Thorgaard (1984) suggesting that UV irradiation is not the only cause of reduced viability in androgenesis in tilapia.

Secondly, it was suggested that the low diploid androgenetic yield might be the result of deleterious effects of physical shocks to inhibit first mitotic division (Gillespie and Armstrong, 1981; Scheerer *et al.*, 1986; Thorgaard *et al.*, 1990; Masaoka *et al.*, 1995). Chromosomal changes such as terminal deletion, exchange type aberration, inter- or intra-arm exchange or inter-chromosome exchanges through rapid cell cycles caused by hydrostatic pressure treatment at the first cleavage were reported in gynogenetic salmon (Yamazaki and Goodier, 1993) and in gynogenetic loach (Suwa *et al.*, 1994; Masoka *et al.*, 1995). These changes are similar to those induced by irradiation (Yamazaki and Goodier, 1993; Yamazaki *et al.*, 1989), ageing or interspecific hybridization (Yamazaki *et al.*, 1989). Deleterious mitotic aberrations, associated with microtubules and centrioles that are affecting the structure and orientation of spindle fibres and poles were observed in fertilized eggs subjected to optimum heat shock treatments for the induction of mitotic gynogenesis (Garcio-Abiado, 1995). Chourrout, (1987) suggested that inhibition of first mitosis may

double the initial number of fragments, which may severely reduce survival. In the present study low survival rates of diploid androgenetic tilapia sharply decreased from 10.69±2.24 % in pigmentation stages to 0.07±0.07 % (relative to the controls) in yolk sac stage under optimal conditions (heat shock for 3.30 at 42.5°C at 25 m.a.f), suggesting that low viability may be brought about by deleterious effects of heat shock treatment affecting aspects of zygote and blastula development rather than karyokinesis. Deleterious effects of heat shock treatment may possibly be more harmful at a later stage of development. Additionally, based on the hypotheses of Markert (1982), homozygosity of androgenetic progeny may cause inviability through disruption of topographic interaction of chromosome of the interphase nucleus. According to Markert (1982), the highly ordered sequence of gene programs in cell differentiation and embryonic development may require a correspondingly precise topographic interaction of chromosomes in the interphase nucleus. The author claimed that such topography would be affected by homozygosity, by aneuploidy, by hybridization and by structurally rearranged chromosomes such as produced by translocations, inversions or duplications and thus interfere with the normal timing and levels of gene transcription during embryonic development.

Thirdly, inbreeding depression arising from homozygosity of deleterious alleles was suggested for the low viability of androgenetic progeny (Gillespie and Armstrong, 1981; Scheerer *et al.*, 1986, 1991; Bongers *et al.*, 1994) as well as mitotic gynogenetic progeny (Yamazaki and Goodier, 1993). However studies of gynogenesis and androgenesis with inbred parents have given conflicting results. Komen *et al.* (1992a) reported that homozygotic male parents yielded significantly more normal and fewer deformed gynogenetic carp fry than heterozygous males. Streisinger *et al.* (1981) found greatly

improved survival among the gynogenetic progeny of homozygous diploid gynogenetic zebra fish. Higher survivals of androgenetic carp were reported using homozygous males (Bongers *et al.*, 1994). On the other hand, Scheerer *et al.* (1986, 1991) could not improve the survivals of androgenetic rainbow trout by using an inbred sperm source. Gillespie and Armstrong (1981) also found no significant improvement in survival among the androgenetic offspring of an androgenetic diploid male axolotl.

Finally, specific female effects on variable survival rates of androgenetic and mitotic gynogenetic fish were reported by several authors (Lou and Purdom, 1984; Nagy, 1987; May *et al.*, 1988; Komen *et al.*, 1991; Diter *et al.*, 1993; Bongers *et al.* 1994 and 1995; Myers *et al.*, 1995a). Quillet *et al.* (1991) argued that genetic factors specific to females could contribute to the viability of their homozygous progenies and thus interfere with the determination of the actual efficiency of treatment as far as survival is concerned. In the present study, a significant female, but not male effect was found on the survival of androgenetic tilapia suggesting that great differences exist between egg batches of certain females in their susceptibility to the UV and diploidization treatment. A similar observation was reported by Myers *et al.* (1995a) who claimed that it is necessary to select broodstock suitable for producing "host" eggs under a specific UV exposure protocol.

It is worth mentioning that a constant incubation temperature during the period between fertilization and starting the shock is also vital so that each batch of eggs receives the shock at the same development stage since a small change in base temperature affects the developmental rate and thus the biological timing of the onset of the shock.

Although very low survival (0.07 % to yolk sac resorption stage, relative to the controls) was obtained during the experiment of optimization of treatment parameters to

induce diploid androgenetic red tilapia under the optimum UV exposure time of 5 min, in one preliminary experiment, a very high survival rate of 22 % (relative to the control, to first feeding stage) was obtained for diploid blond androgenetic tilapia using 4.5 min UV exposure time and 4 min heat shock applied at 27 m.a.f. It is not easy to give a definite explanation but a strong female specific effect could be responsible for this phenomenon.

Multilocus DNA fingerprinting was successfully used to verify all-paternal inheritance. It has been shown that the hypervariable 33.15 DNA probe (Jeffreys *et al.*, 1985) can be used to produce individual-specific DNA fingerprints from tilapia. The DNA fingerprint result showed relatively low variability. Carter *et al.* (1991) reported similar observations and naturally low levels of variation or inbreeding under laboratory conditions were suggested as reasons for low variability. The application of the DNA fingerprinting technique for verification of all-paternal inheritance in androgenesis is more clearcut than verification of maternal inheritance since it is difficult to distinguish meiotic and mitotic gynogenetics (Carter *et al.* 1991; Sarder, 1998). A number of DNA probes are now available and each of them has potential to produce individual specific DNA profiles. DNA fingerprinting techniques have been successfully used to confirm all paternal inheritance (Nagoya *et al.*, 1996; Sarder, 1998) and all maternal inheritance (Carter *et al.*, 1991; Han *et al.*, 1992; Takagi *et al.*, 1995; Eenenaam *et al.*, 1996; Young *et al.*, 1996; Corley-Smith *et al.*, 1996; Sarder, 1998).

The results of progeny testing of androgenetic males supported the viability of "YY" male in blond and red tilapia. The viability of "YY" males in fish appears to vary between species. The progeny testing of androgenetic rainbow trout males showed the viability of YY males as well as the occurrence of XX males which might reflect autosomal genes or

environmental influences affecting sex determination (Parsons and Thorgaard, 1985; Scheerer *et al.* 1991). The percentage of male offspring of androgenetic red and blond males crossed with wild type females was 100 % (for 13 males) and 84 % (for 1 male) and significantly differed from the expected sex ratio (1:1) ($P<0.01$-0.001) in the present study. The occurrence of 16% females could be explained by other genetic sex-modifying factors or/and environmental effects on sex determination. Although female homogamety and male heterogamety were proposed by using gynogenetic technique in Nile tilapia (Penman *et al.*, 1987; Shah, 1988), Mair *et al.* (1991a) proposed a monofactorial system with rare autosomal recessive genes epistatic to the major sex determining gene. The presence of a rare secondary sex-determining loci (SDL-2) in tilapia also has been suggested by Hussain *et al.* (1994) and Chapter 3 in this thesis. A secondary sex-determining gene has been described in mitotic gynogenetic common carp (Komen *et al.*, 1992b). Furthermore the present study showed that sex ratios of androgenetic tilapia did not differ from the expected sex ratio of 50 % female and 50 % male ($P>0.05$) strongly supporting that Nile tilapia has a homogametic female and heterogametic male system (Penman *et al.*, 1987; Shah, 1988; Mair, 1991a).

The attempt to produce androgenetic red and blond YY neofemales using oral administration of DES hormone was not successful. Although two of the DES-treated females tested with an XX neomale showed XX genotype, no viable eggs were obtained from the other DES-treated androgenetic tilapia. Similar observation were reported that homozygous androgenetic female rainbow trout showed poor quality eggs (Scheerer *et al.*, 1991). The reason might be deleterious effects of inbreeding on reproductive system. Kincaid (1976: cited in Scheerer *et al.*, 1991) noted reduced hatchability of eggs and increased frequencies of crippled fry in crosses involving female trout which were produced

from two generations of sib-matings. Another reason could be that the optimum treatment of 1000 $mg.kg^{-1}$ DES for 10 days in tilapia (Mair and Santiago, 1994) might not be suitable for feminization of androgenetic tilapia resulting in intersex fish which were not able to produce any gametes. Although there is no report for feminization of androgenetic tilapia, feminization using DES hormone successfully produced YY neofemales in tilapia (Mair *et al.*, 1997; Tuan, 1997; Chapter 5 in this thesis). A higher dosage of DES might be needed for successful feminisation of androgenetic tilapia.

Androgenesis is a valuable technique to analyse colouration as well as sex determination mechanism in fish. Scott *et al.* (1987) and McAndrew *et al.* (1988) first reported the autosomal recessive blond and autosomal dominant red skin characters, respectively. In the present study, blond and red androgenetic tilapia showed paternal coloration as expected and this supports the conclusion that blond coloration is autosomal recessive whereas red coloration is autosomal dominant to wild type coloration. Androgenetic red tilapia produced in this work showed variable black blotching pattern, which is unusual for the homozygous state of the red gene. Black blotched tilapia (0-26.6 % of the fish's surface) were produced as result of crosses between wild type (rr) and homozygous red (RR) tilapia and their inheritance is still not fully understood. According to McAndrew *et al.* (1988), the blotched phenotype is epistatic to the red gene and can only be expressed in its presence. On the other hand their results also suggested that blotching might be controlled by a single gene with two alleles B and b, where B might be responsible for blotching and b for unblotched character. The present study agreed with that theory. Red colour patterns from the further generations of androgenetic red tilapia crossed to different females will be discussed in Chapter 5.

Chapter 3:

Recombination rates of skin colouration, allozyme and sex determining loci in meiotic gynogenetic Nile tilapia, *O. niloticus*

3. Recombination rates of skin colouration, allozyme and sex determining loci in meiotic gynogenetic Nile tilapia, *O. niloticus*

3.1. Introduction

Gynogenesis is a genome manipulation technique in which eggs are fertilised with denucleated sperm so that the resulting embryo shows no paternal inheritance. Denucleated sperm only triggers the embryonic development of eggs without any genetic contribution. The embryos produced in this way possess a single set of maternal chromosomes (haploid syndrome) and thus die before hatching. A variety of physical shocks (temperature or hydrostatic pressure) permitting the retention of the second polar body or inhibition of the first mitotic division during embryonic development of eggs, results in diploid meiotic and mitotic gynogenetics, respectively (see reviews by Purdom, 1983; Chourrout, 1987, Ihssen *et al.*, 1990 and section 2.1.1. in this thesis). Induced meiotic gynogenesis has been successfully applied to several fish species. Table 2.1. shows the published reports on meiotic gynogenesis in different fish species.

One of the major interests for meiotic gynogenesis technique was the rapid production of inbred lines. However, production of inbred lines using meiotic gynogenesis is not as rapid as was anticipated due to the rate of gene-centromere crossing over and chiasmata interference during meiosis I (Thompson, 1983; Allendorf and Leary, 1984; Nagy and Csanyi, 1982). The application of electrophoretic and morphological markers shows that the rate of crossing over in the first meiotic metaphase produces high levels of heterozygosity in chromosome regions distant from the centromere (Purdom,

1976; Nagy and Csanyi, 1982; Thorgaard *et al.*, 1983; Guyomard, 1984; Gervai and Csanyi, 1984; Allendorf *et al.*, 1986; Naruse *et al.*, 1988; Hussain *et al.*, 1994; Guo and Allen, 1996).

On the other hand, isogenic lines (genetically identical but not homozygous at every locus) can be produced more rapidly from meiotic gynogenetic individuals than conventional sibmating, which requires many generations. Isogenic lines can be used for studying genetic versus environmental effects on fish e.g. growth trials (Mair, 1993). Han *et al.* (1991) suggested that using meiotic gynogenesis even if meiotic gynogenesis is repeated for several generations would never produce homozygous inbred lines. However, meiotic gynogenesis is a very useful technique for estimating gene-centromere recombination rates for gene mapping (Nagy and Csanyi, 1982; Thorgaard *et al.*, 1983; Hussain *et al.*, 1994). Several estimations of gene-centromere rate have been reported by meiotic gynogenesis at several isozyme loci in rainbow trout (Thorgaard *et al.*, 1983; Thompson and Scott, 1984; Guyomard, 1984; Allendorf *et al.*, 1986), in brown trout (Guyomard, 1986), in *Salvelinus* species (Arai *et al.*, 1991), in common carp (Taniguchi *et al.*, 1986; Linhart *et al.*, 1987), in catfish (Liu *et al.*, 1996), in plaice (Purdom, 1976; Thompson, 1983), in paradise fish, *Macropodus opercularis* (Gervai and Csanyi, 1984), Nile tilapia (Mair, 1993; Hussain *et al.*, 1994), in mollusc, *Mulinia lateralis* (Guo and Allen, 1996) and in abolone, *Haliotis diversicolor diversicolor* and *H. discushannai* (Fujino *et al.*, 1989, 1992). Gene-centromere mapping is a useful technique to obtain information about the meiotic system, comparative and evolutionary studies, and estimation of inbreeding rate in fish species (Thorgaard, 1983; Allendorf *et al.*, 1986). Study of the recombination rate between each gene and its centromere can provide better

understanding of genetic mechanisms involved in morphological characters such as skin colour (Nagy *et al.*, 1978; Guyomard, 1984; Streisinger *et al.*, 1986; Hussain *et al.*, 1994).

Another application of diploid gynogenesis is the production of monosex populations in fish with female homogamety (XX) (Golovinskaya, 1969; Stanley, 1976; Nagy *et al.*, 1978; Refstie *et al.*, 1982; Na-Nakorn, 1995; Pongthana *et al.*, 1999). The sex ratio of gynogenetic offspring can also provide information about sex determination mechanisms in fish. The induction of gynogenesis has been used to elucidate sex determining mechanisms in *Oreochromis* species (Penman *et al.*, 1987; Shah, 1988; Avtalion and Don, 1990; Mair *et al.*, 1991a, b; Hussain *et al.*, 1994), common carp (Komen *et al.*, 1992b), salmonids (Chourrout and Quillet, 1982; Refstie *et al.*, 1982), zebra fish (Streisinger *et al.* 1981) and plaice (Purdom, 1976). Hormonal sex reversal of gynogenetic females to males and crossing of these neomales to normal females can ensure the production of genetically all female populations. When mitotic gynogenesis is applied to neofemales (XY), male progeny will be YY males which can be used for all male production with normal females (Varadaraj and Pandian, 1989; Mair *et al.*, 1993). The direct production of single sex populations of fish does not appear to be a viable approach as significant levels of recombination between sex determining loci can result in mixed sex progeny groups in tilapia (Avtalion and Don, 1990; Mair *et al.*, 1991a,b; Hussain *et al.*, 1994; Muller-Belecke and Horstgen-Schwark, 1995) and in common carp (Komen *et al.*, 1992b).

In the present study, diploid meiotic gynogenesis was used to estimate gene-centromere recombination rates for the *ADA**, red skin colour and a putative secondary

sex-determination locus in red Nile tilapia. Possible linkage between the red colouration and putative secondary sex determination loci was analysed. DNA samples were also taken from these fish to provide reference families for a collaborative genome-mapping programme.

3.2. Materials and methods

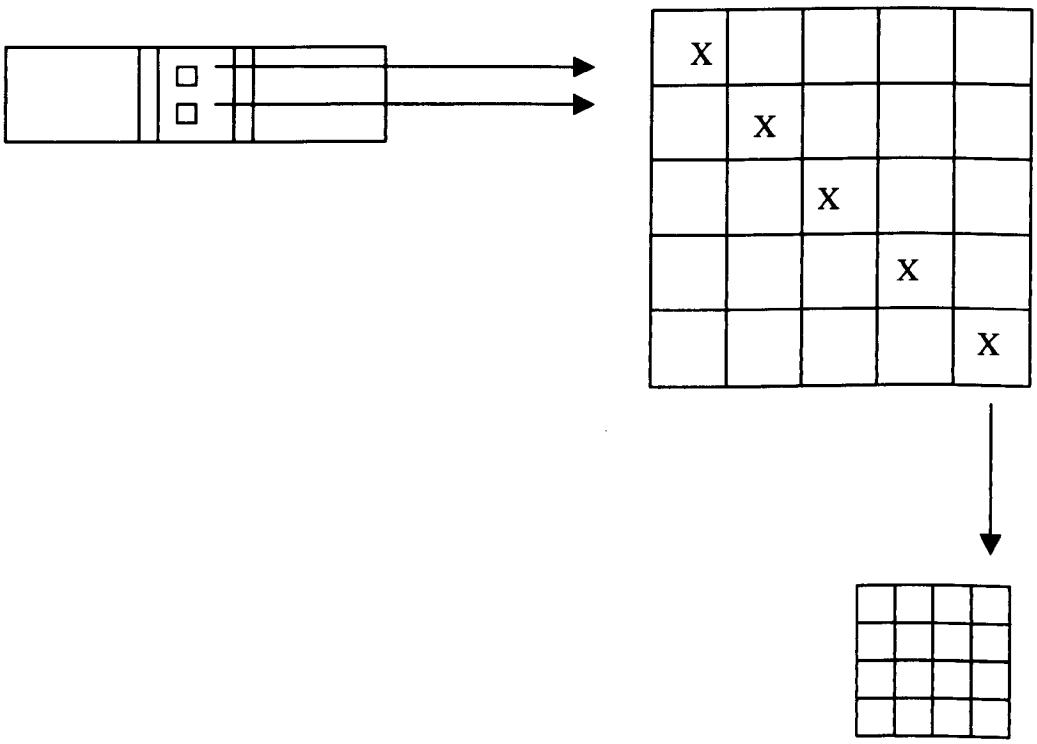
3.2.1. Source of broodstock

The red *O. niloticus* brood stock used for this study were descended from an electrophoretically tested, pure stock of the Tilapia Reference Collection maintained at the Institute of Aquaculture, University of Stirling, Scotland (Section 2.1.1) and attention was given to their maintenance (Section 2.2.1.1 and section 2.2.1.2.) and feeding (Section 2.2.1.3) as previously described.

3.2.2. Collection and ultraviolet irradiation of milt

Collection and UV irradiation of milt were carried out according to Hussain (1992) with some minor modification. Fresh milt was collected from sexually mature males for every experiment by artificial stripping. Before stripping the urine was first ejected and faeces or mucus were cleaned from the genital papilla with a paper towel. Milt was drawn into a clean micro-pipette by capillary attraction when it was placed at the opening of the uretha, then drained into a clean 1.5 ml microtube and stored at 4°C until further use. Milt used for UV treatment in all gynogenetic experiments was first checked for motility by adding a drop of water to 1 µl of milt on a glass slide under a light microscope. If non-motile sperm were more than 10 % in the sample, it was not used and a new batch of milt was collected. Sperm counting was done using a haemocytometer (depth of the chamber: 0.1 mm and area of the 16 square groups: 0.04 mm^2) as follows: Firstly, 10 µl of milt was added to 490 µl of Modified Fish Ringers solution, pH 8.0 (Appendix 2.1.) in a microtube to make the total volume of 500 µl and

mixed. Secondly, 10 µl of diluted milt from the first tube was added to another microtube containing 90 µl of Modified Fish Ringers solution to give a final volume of 100 µl. The final dilution of milt was well mixed before 10 µl of diluted milt was placed on the haemocytometer under a coverslip. After the sperm had settled, they were counted in 5 large squares as indicated below on each side of the haemocytometer. An average was calculated by counting the sperm on both sides of the haemocytometer to minimise error during counting.



Assuming that the total number of sperm in 5 large squares on one side was 150 and the other side was 200, to find the actual number of sperm needed to obtain the optimal concentration of $2.5*10^7$ ml-1, a calculation was done as follows.

Average number of sperm in 5 large squares = (150+200)/2=175

Average number of sperm in a small square = 175/80=2.19

Total number of sperm = 2.19 x $\frac{(4000 \times 1000)}{\text{chamber volume}}$ x $\frac{(50 \times 10)}{\text{dilution factor}}$ = 4.38×10^9 ml^{-1}

Amount of milt needed = $(2.5 \times 10^7)/(4.38 \times 10^9) \times 1000$ = 5.7 µl of undiluted milt

Based on the calculated sperm count, 2x5.7 µl of milt were diluted with 1988.6 µl of Modified Fish Ringer Solution in a 30 mm Petri dish for irradiation. The Petri dish containing milt and Modified Fish Ringer solution was gently stirred and irradiation was carried out at 4°C using a 254 nm wave length UV lamp (Ultra-Violet Products, San. Gabriel, Calf.) at a dose of 250-265 μWcm^{-2} for 2 min. The UV intensity was measured by a radiometer (Ultra-Violet Products Inc.). Irradiated milt was immediately used to fertilise eggs.

3.2.3. Egg collection and fertilisation

Eggs were collected by gently stripping from an ovulated female (Section 2.2.4) After stripping, each batch of eggs was fertilised *in vitro* either with normal or UV treated milt.

3.2.4. Heat shock

A temperature controlled water bath (Jencons Scientific Ltd) was used for heat shock treatments (Section 2.2.6). The water bath containing overnight aerated clean tap water was warmed to 42±0.1°C before collecting the milt and eggs. Five minutes after

fertilisation with UV irradiated milt, eggs were moved to the water bath for 4 min. in a nylon mesh tea strainer. One batch of eggs fertilised with UV irradiated milt was not heat shocked and retained as a haploid control while another batch of eggs were fertilised with normal milt as a diploid control group.

3.2.5. Experimental design for production of gynogenetic tilapia

In order to produce meiotic gynogenetic tilapia, several presumably heterozygous red female broodstock originally generated from crosses between a female and two males (Keith Ranson personal communication) in the same age group were transferred to the glass aquarium system (Fig. 2.1) and P.I.T. tagged (Section 2.2.3). The origin of the dominant red colour variant and description of the homozygous and heterozygous phenotypes are given in McAndrew *et al.* (1988).

Induction of meiotic gynogenesis was described in Section 3.2.3 and 3.2.4. A single blond or red male was used in a single pair mating to ensure having a high sperm motility in each experiment.

3.2.6. Egg incubation and checking of survival rates

All treated and untreated batches of eggs were identically incubated (Section 2.2.4.1. and 2.2.9) and the survival rate of embryos in each group was counted at pigmentation (40-42 hours after fertilisation), hatching (80-90 hours after fertilisation) and yolk sac resorption stages (9-10 days after fertilisation). The number of normal and deformed fry at both hatching and yolk sac stages were recorded.

3.2.7. Determination of ploidy

The ploidy of all treatment and control batches was determined by chromosome preparation (Section 2.2.10) from a sub sample of hatched or one day old larvae. Haploid and diploid metaphases were composed of one (N=22) or two (N=44) sets of chromosomes respectively (Fig.3.1).

3.2.8. Scoring of progeny phenotypes

At the hatching stage, it was difficult to differentiate body colour pattern, therefore phenotype scoring was carried out at first feeding stage by using a binocular dissecting microscope (without harming the fry). The body colour of progeny was categorised as "red" and "wild type" and were counted individually at this stage.

3.2.9. Fry rearing and on-growing

The gynogenetic fry were transferred to an early rearing system (Section 2.2.1.1.1) at first feeding stage, and reared further in an advanced fry rearing system (Section 2.2.1.1.2) and stocking system (2.2.1.1.3). The fish were fed according to methods described early in section 2.2.1.3. The fish were grown to 4-10 months of age.

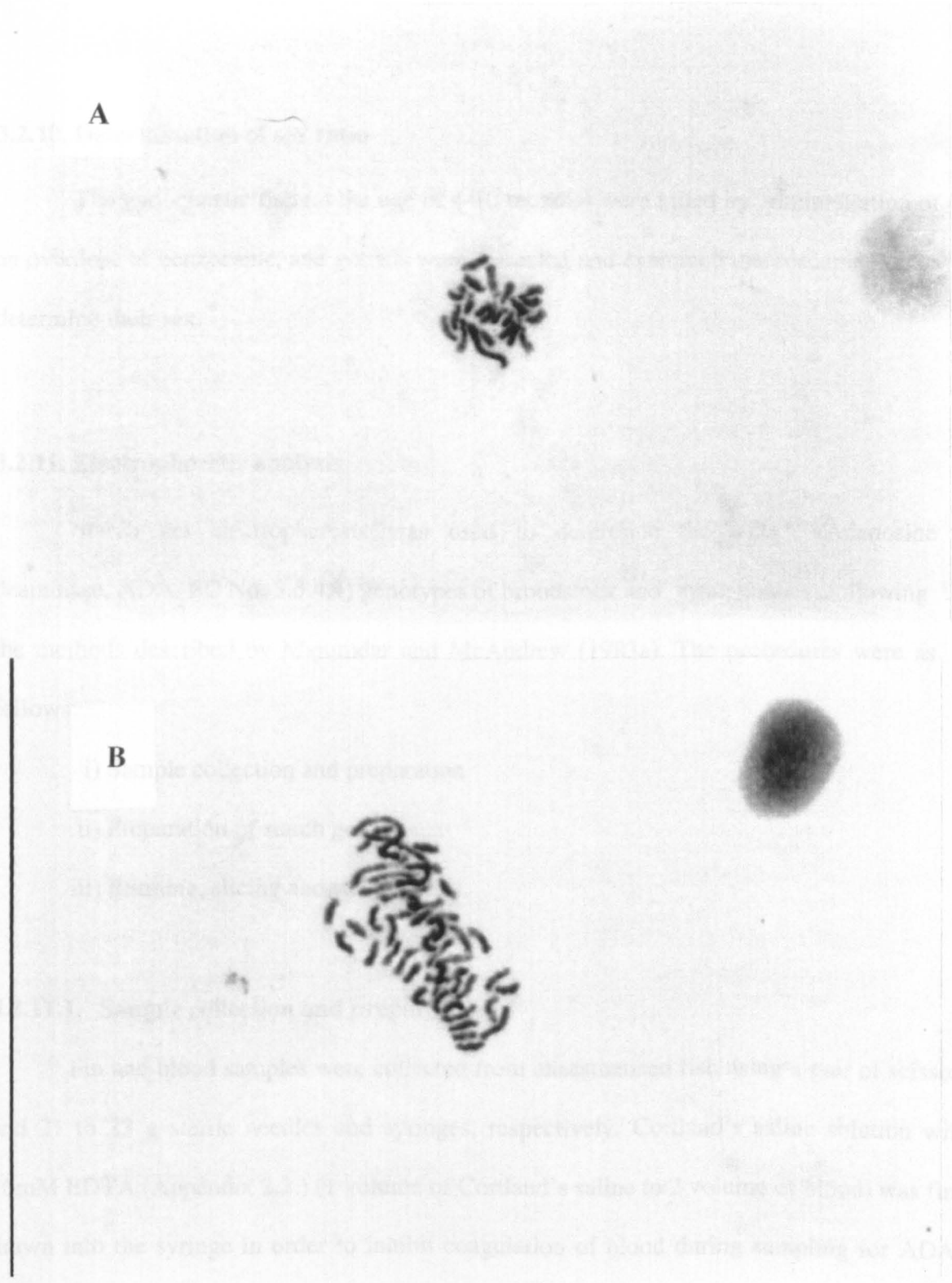


Figure 3.1. Metaphase chromosome spread of gynogenetic A. haploid (N=22) and B. diploid (N=44) embryos of *O. niloticus*.

3.2.10. Determination of sex ratio

The gynogenetic fish (at the age of 4-10 months) were killed by administration of an overdose of benzocaine, and gonads were dissected and examined macroscopically to determine their sex.

3.2.11. Electrophoretic analysis

Starch gel electropheresis was used to determine the *ADA** (Adenosine deaminase, ADA. EC No: 3.5.4.4) genotypes of broodstock and gynogenetics, following the methods described by Majumdar and McAndrew (1983a). The procedures were as follows:

i) Sample collection and preparation

ii) Preparation of starch gels

iii) Running, slicing and staining gels.

3.2.11.1. Sample collection and preparation

Fin and blood samples were collected from anaesthetised fish using a pair of scissors and 21 to 23 g sterile needles and syringes, respectively. Cortland's saline solution with 10mM EDTA (Appendix 2.2.) (1 volume of Cortland's saline to 2 volume of blood) was first drawn into the syringe in order to inhibit coagulation of blood during sampling for ADA* and DNA. Blood samples were immediately mixed well with the anti-coagulant in the syringe and transferred to 1.5 ml sterile micro tubes, which were then centrifuged at 1400 g for 2 mins and the supernatant removed. The pelleted blood and fin samples then were stored at –20°C until further use.

Samples were thawed for a few minutes and cytoplasm from the thawed tissue was then absorbed onto 3-4 x 12 mm pieces of Whatman No.1 filter paper placed onto the sample in the tubes for electrophoresis.

3.2.11.2. Preparation of starch gel

12% starch gel was prepared using 22 g starch (Sigma Ltd.) mixed with 220 ml of diluted Tris-EDTA-Borate (TBE) buffer (see Appendix 2.3.) in a Buchner flask. The mixture was heated with constant rotation of the flask to an almost translucent jelly state, quickly degassed using a vacuum water pump and then poured into 6 mm thick gel frames. The gel, covered with a glass plate, was allowed to set and cool overnight at room temperature.

3.2.11.3. Running, slicing and staining gels

The gel was taken out of the frame and a parallel cut was made 3 cm from the edge to create a point of origin. The samples (filter paper) were placed along this cut with about 25-30 samples per gel and one tracking dye (0.1 % bromophenol blue) at each end of the gel to indicate mobility through the gel. When all samples were correctly arranged, the frame was placed back on the gel and a perspex spacer positioned between the gel and frame to keep the sample slot closed (to keep the sample tight).

The gel was then placed in an electrophoretic bath with 1x TBE buffer. A gauze wick soaked in the buffer was applied to either end of the gel to connect the gel and buffer. The gel was then covered with a polythene sheet to reduce evaporation and ice in a plastic bag was placed onto the polythene sheet to prevent warming of the gel. The bath tray was covered with a transparent lid and the gel was allowed to run for 15 mins with an electrical

current of 150-200 V, at 4°C in a refrigerator. Following the initial running, the filter papers were removed and the gel was run again for 3-4 hrs at same current. The gel was taken from the refrigerator and removed from the bath. It was then sliced horizontally into three slices, each of which could be stained for a different enzyme system but in the present study only Adenosine deaminase (*ADA**) was examined. The appropriate stains (Appendix 2.4.) for the *ADA** were weighed and mixed with staining buffer solution and 2 % agar (at approximately 50-60 °C). This mixture was poured over the slice, allowed to set and then incubated at 37°C until the banding patterns became visible. The electropherograms were then analyzed and scored for the respective genotypes and when necessary they were preserved in gel fixative solution (Appendix 2.5.). Finally, they were dried onto filter paper for storage.

3.2.12. Statistical analyses

The survival rates of meiotic gynogens, their respective diploid and haploid controls were tested by the non-parametric Kruskal Wallis test. All data were transformed to arc-sine for statistical analyses. The results were presented as mean and standard error of mean (±SE). All statistical analyses were performed by Minitab 9.2 software.

Recombination frequencies for the red colour and *ADA** loci were estimated using the equation described by Nace *et al.* (1970). Recombination frequency between a locus and its centromere can be estimated from the proportion of heterozygotes (y) in the diploid gynogenetic progeny obtained from a heterozygous female. However, it is difficult or impossible to distinguish the heterozygotes red (Rr) individuals from the homozygotes red (RR) individuals. Therefore assuming that the proportions of the

homozygotes for RR and wild type alleles (rr) are identical. Therefore proportions of heterozygotes (Rr) can be calculated from the following equation;

Proportion of heterozygotes (y)= (No of red - No of wild type)/Total No of meiogynes

The proportion of heterozygotes for *ADA** locus can be obtained from the following formula since the heterozygotes can be directly visualised on the gel.

y= (No of recombinants at *ADA** locus) / (Total no of meiogynes)

Heterogeneity of colour and sex distribution of offspring between females and within the experiments from each female was performed using 2 x c contingency test (significant level $P<0.05$) (Bailey, 1981).

The analyses for joint segregation or linkage of red and sex determination locus were made by using a G test of independence (two-way tables) (Sokal and Rohlf, 1987). The G test is recommended in preference to χ^2 whenever the observed frequency minus the expected frequency is less than the expected frequency for any cell. Both methods commonly result in the same conclusion. When they do not, many statisticians prefer G and recommend its routine use.

The sex ratios of diploid controls were analysed by Chi-square test, comparing with 1:1 sex ratio.

3.3. Results

3.3.1. Survival rates of meiotic gynogenetics

Mean survival rates at pigmentation, hatching and yolk sac resorption stages of diploid controls, haploid controls and meiotic gynogenetics are presented in Table 3.1. All haploid control batches showed a reduction in embryonic survival from the pigmentation stage and they were usually dead at hatching or before yolk-sac resorption. The fry in the haploid control group showed a typical haploid syndrome with a twisted body and enlarged pericardium. The mean survival rates of the haploid control groups at pigmentation and hatching stages were 33.36±5.48% and 4.47±1.65% (relative to the diploid controls), respectively, significantly lower than the diploid controls for both developmental stages ($P<0.05$). The chromosome study showed a single set (N=22) of chromosome in haploid groups and double set (N=44) of chromosomes in diploid gynogens and diploid control groups (Fig. 3.1).

The use of heat shock to induce meiotic gynogenesis by suppression of the second meiotic division was successful. Meiotic gynogenesis produced normal diploid individuals with higher survival rates at hatching and yolk sac resorption stage than haploid gynogenesis. The survival rates of diploid controls and diploid meiotic gynogens varied between females and even experiments using the same females. At pigmentation, hatching and yolk sac stages the mean survival rates of diploid controls were 55.00±2.66%, 47.77±2.98% and 39.09±3.08% while the mean survival rates of diploid meiotic gynogens were 44.00±5.19%, 28.70±4.51% and 19.59±3.58% (relative to the diploid controls), respectively. The mean survival rates of diploid controls and diploid meiogynes were significantly different at all

Table 3.1. Mean survival (%±SE) of diploid control (C), haploid control (HC) and meiogynes (MG) of heterozygous red (Rr) tilapia, *O. niloticus* at pigmentation, hatching and yolk sac resorption stages in different experiments. Common superscript is not significantly different at each developmental stage (R: Relative to controls, S1: Sire no 1, S2: Sire no 2).

Sire		Pigmentation			Hatching			Yolk sac resorption	
No	Female tag no.	C	HC	MG	C	HC	MG	C	MG
1	002 040 779	59.84±3.5	10.14±7.08	22.18±3.08	55.2±4.96	0.00	16.10±51.31	52.25±4.01	10.51±1.38
1	015 316 612	60.99±6.96	18.24±11.09	37.85±11.12	56.65±7.73	4.40±4.41	24.26±10.80	49.11±11.46	7.13±2.73
1	011 554 623	43.05±9.63	5.95±5.97	22.64±7.88	39.97±8.35	0.00	11.28±7.46	30.99±1.38	2.97±0.93
1	014 523 892	60.25±8.70	5.96±0.70	25.83±1.73	40.50±24.78	0.00	8.17±1.39	24.21±9.60	3.20±0.91
1	011 620 585	62.03±4.73	28.43±7.18	32.30±6.15	51.17±4.15	4.60±1.90	18.25±5.16	41.78±5.54	13.76±4.99
Mean S1		58.76±3.01	17.96±4.22	29.91±3.46	49.88±3.69	2.72±1.19	16.89±3.09	40.86±3.84	9.15±2.28
2	012 526 884	48.11±15.86	1.63±1.63	21.51±1.85	39.65±20.63	0.48±0.48	6.67±1.00	35.90±21.49	4.45±1.12
2	011 113 094	46.57±11.18	35.46±11.91	12.10±3.95	37.35±7.95	1.97±1.98	4.56±2.64	32.23±8.10	3.55±2.24
2	011 895 051	60.05±13.83	15.31±5.05	13.13±6.32	57.25±12.94	0.00	10.86±4.96	53.89±12.29	6.99±3.27
2	013 559 035	42.38±12.42	10.82±0.37	9.52±5.83	37.79±12.28	0.00	5.25±2.71	22.99±2.57	1.82±0.09
2	010 602 500	59.63±4.96	22.57±6.73	16.17±4.13	51.02±5.21	2.15±2.15	9.38±4.48	37.21±4.93	5.85±2.05
	Mean S2	51.35±4.72	17.16±4.38	14.48±2.08	44.61±5.07	0.92±0.54	7.34±1.40	36.44±5.19	4.53±0.91
Mean (S1 and S2)		**55.80±2.66** [b]	**17.64±3.02** [a]	**23.74±2.68** [a]	**47.77±2.98** [c]	**2.00±0.76** [a]	**13.07±2.13** [b]	**39.09±3.08** [b]	**7.30±1.47** [a]
Mean (S1 and S2) (R)		**55.00±2.66**	**33.36±5.48**	**44.00±5.19**	**47.77±2.98**	**4.47±1.65**	**28.70±4.51**	**39.09±3.08**	**19.59±3.58**

developmental stages ($P<0.05$). A significant drop in survival between pigmentation, hatching and yolk-sac stages were found in all groups with an overall survival pattern of diploid control>diploid meiogyne>haploid control.

The survival rates of diploid control, haploid control and meiotic gynogenetics at each developmental stage were also grouped according to the sire of females used in these experiments. There were no significant differences between the survival rates of diploid and haploid controls derived from sire 1 and sire 2 at any developmental stages ($P>0.05$). Although no significant differences were found between the survival rates of meiotic gynogens derived from sire 1 and sire 2 at yolk sac resorption stage ($P>0.05$), there was a significant differences between the survival rates of meiotic gynogens from sire1 and sire 2 at pigmentation and hatching stages ($P<0.05$).

3.3.2. Recombination rates in meiotic gynogens for red skin coloration

The meiotic gynogenetics were produced from 10 heterozygous (Rr) female broodstock. The colour genotype of each female was determined by crossing with a wild type male. The resulting offspring's colour segregated into 1 red: 1 wild type indicating that the mother was heterozygous (Rr).

The original cross producing the heterozygous red female broodstock used in this study was between a female and two males. The banding patterns of the individuals used for producing meiotic gynogens were prepared using multilocus DNA fingerprints and analysed using the Geldoc ID advanced system (UVP version 3.01, 1997). A dendogram was produced using the neighbour joining algorithm of Saitou and Nei (1987) based on the percentage of

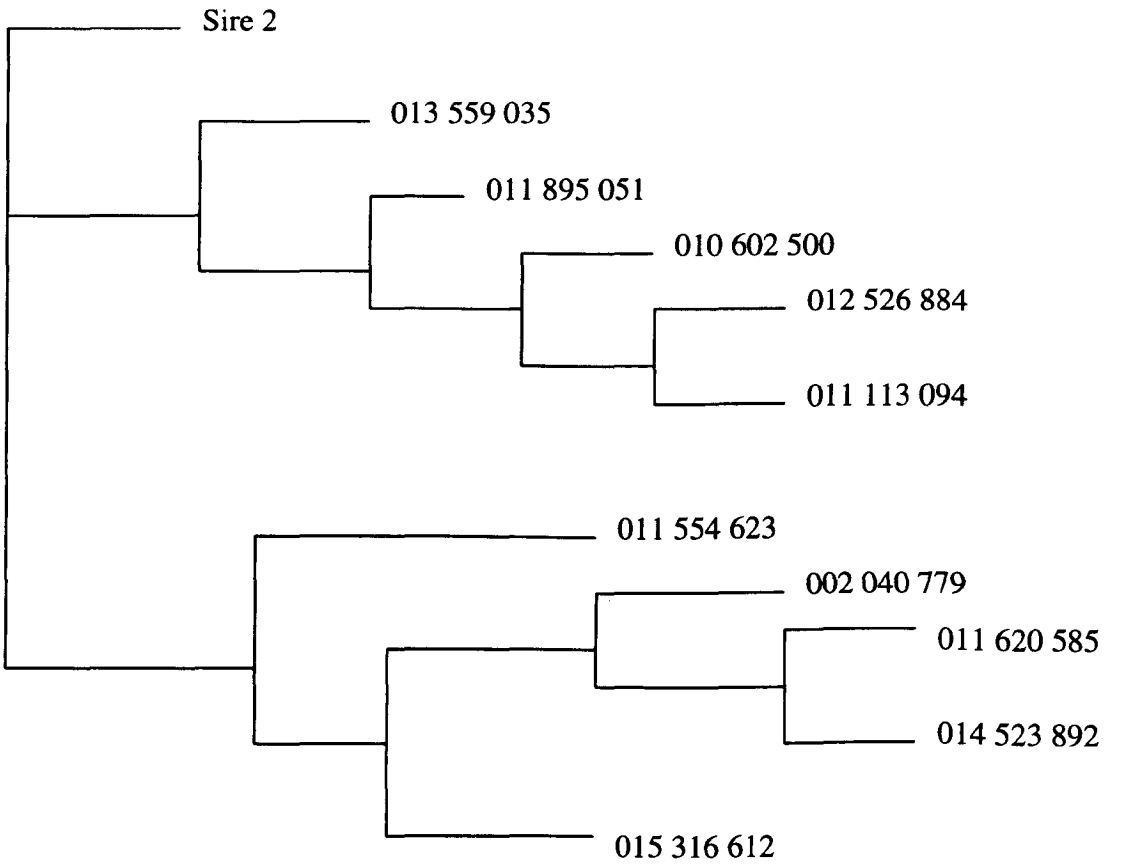


Figure 3.2. Dendogram showing the relationship between the females based on multilocus DNA fingerprinting using Jeffrey's 33.15 probe.

bands shared. This result showed that the females 002 040 779, 015 316 612, 011 554 623 and 014 523 892 might belong to one of the sires (1) while the females 012 526 884, 011 113 094, 011 895 051, 013 559 035 and 010 602 500 might be the offspring of the other sire (2) (Fig. 3.2).

The distribution of body colour pattern and the calculated frequency of recombinant heterozygotes in a total of 46 gynogenetic trials involving 10 different heterozygous (Rr) red

females are presented in Table 3.2. Contingency test analysis was practised to test differences within experiments of each female and between pooled progeny of all females for the distribution of red and wild type phenotypes. The only significant deviation was among the progenies of female 015 316 612 while the pooled progenies of the other 9 females showed homogeneity for the distribution of red and wild type. The contingency test analysis of each experiment (total 4 experiments) derived from the female 015 316 612 showed heterogeneity. The contingency test analysis showed homogeneity when the experiment no: 2 from the female 015 316 612 was excluded indicating that heterogeneity occurred in the experiment no: 2. Therefore, it can be assumed that 1 out of 46 experiment may not reflect any real heterogeneity. When the number of wild type meiotic progeny exceeded the number of red meiotic progeny in a given experiment, recombination rate (y) value was assumed to be zero i.e. no recombination between the gene and its centromere.

The proportion of heterozygous red (y) ranged between 0.000-0.447 when the recombination frequency of experiment no:2 showing heterogeneity from the female 015 316 612 was included. However, when this recombination frequency was excluded the proportion of heterozygous red (y) ranged between 0.000-0.275. The y value of 0 suggests that in such cases there was no crossover between the gene and its centromere, therefore all red meiogynes were presumably homozygous for the "R" allele while all wild type meiogynes were homozygous for the "r" allele.

The mean recombination frequencies of sire group 1 and 2 were 0.154±0.076 and 0.082±0.047, respectively. However, when the recombination frequency of experiment no:2 from the female 015 316 612 was excluded, the recombination frequency of sire 1 group was 0.120±0.044. One-way ANOVA result showed that there was no significant differences

Table.3.2. Distribution of body colour pattern and frequency of red heterozygotes in meiotic gynogenetic tilapia, *O. niloticus*, progeny derived from 10 red heterozygous females (Rr) at first feeding stage (10-11 days after fertilisation).

Sire no.	Female tag no.	Experiment no.	Progeny phenotypes		[2]Proportion of heterozygotes(y)	Mean(±SE)
			Red	Wild type		
1	[1]002 040 779 (n.s.)	1	12	3		
		2	52	53		
		3	88	75		
		4	37	28		
	Total		**189**	**159**	**0.086**	
1	[1]015 316 612***	1	3	12		
		2***	51	9		
		3	45	21		
		4	10	0		
	Total		**110**	**42**	**0.447**	
1	[1]011 554 623 (n.s.)	1	3	2		
		2	3	6		
		3	7	10		
		4	46	49		
		5	10	10		
		6	18	14		
	Total		**87**	**91**	**0.000**	
1	[1]011 620 585 (n.s.)	1	2	0		
		2	2	1		
		3	144	103		
		4	58	57		
		5	58	57		
		6	92	74		
		7	48	18		
		8	45	40		
		9	21	18		
		10	79	56		
	Total		**549**	**424**	**0.129**	
						0.154±0.076
1	[1]014 523 892 (n.s.)	1	4	3		
		2	20	17		
		3	16	12		
	Total		**40**	**32**	**0.111**	
2	[1]013 559 035 (n.s.)	1	2	2		
		2	1	2		
		3	9	6		
		4	6	3		
		5	6	1		
	Total		**24**	**14**	**0.263**	

Table 3.2. continued

Sire no.	Female tag no.	Experiment no.	Progeny phenotypes Red	Wild type	[2]Proportion of heterozygotes(y)	Mean(±SE)
2	[1]011 895 051(n.s.)	1	1	1		
		2	2	2		
		3	7	9		
	Total		**10**	**12**	**0.000**	
2	[1]010 602 500 (n.s.)	1	15	16		
		2	6	3		
		3	2	2		
		4	4	5		
	Total		**27**	**26**	**0.019**	
2	[1]012 526 884 (n.s.)	1	6	6		
		2	32	27		
		3	15	15		
	Total		**53**	**48**	**0.050**	
	[1]011 113 094 (n.s.)	1	3	3		
		2	27	22		
		3	4	6		
		4	8	5		
Total		**42**	**36**	**0.077**		**0.082±0.047**
						0.118±0.04

[1]Contingency test results of red and wild type phenotype distribution between offspring from each brood were shown as n.s. (not significant) and *** (significant at P<0.001).

[2]Proportion of heterozygotes (y): (No. of reds- No. of wild type)/Total

between the mean recombination frequencies derived from the sire group 1 with or without the recombination frequency of experiment no:2 and sire group 2.

The mean recombination frequency including all heterozygous red females was 0.118±0.04 and 0.101±0.031 including and excluding the recombination frequency of experiment no:2 from the female 015 316 612, respectively. One-way ANOVA result showed that there was no significant differences between the mean recombination frequencies calculated whether the data from 015 316 612 were included or excluded from the recombination frequency data of this experiment.

3.3.2. Sex ratios of meiotic gynogens and linkage analysis between colour and sex determining locus

The observed sex ratio in pooled diploid control and meiotic gynogens derived from 9 red heterozygous (Rr) females were presented in Table 3.3a and b. The sex ratio of pooled progenies from control crosses was not significantly different from expected 1:1 (Male:Female) ratio from these crosses. However, males were observed among the meiotic gynogens derived from the females 002 040 779, 011 620 585, 012 526 884, 011 113 094 and 015 316 612. The meiotic gynogenetic males showed red phenotype except one male having the wild type phenotype derived from the female 011 620 585.

The analysis of red colouration and sex in these meiogynes showed that there was a strong indication of a linkage between them in progenies derived from the females 002 040 779, 011 620 585 and 012 316 612 . Since only red meiogynes observed in the progenies from the female 015 316 612, linkage analyses could not be performed for this female. Although this female had produced wild type meiogynes at the first feeding stage (Table 3.2), all the

Table. 3. 3a. Linkage analyses between red and sex determining locus in meiotic gynogenetic red tilapia, *O. niloticus* (4-10 months old). Non random associations of genotypes were tested using a G test. (RF: Red female, WTF: Wild type female, RM: Red male, WTM: Wild type male, $^{**}P<0.01$, $^{***}P<0.001$)

Female tag no.	Total experiment no	Progeny phenotype				G
		RF	WTF	RM	WTM	
002 040 779	3	59	66	9	0	12.16^{***}
011 620 585	5	124	119	35	1	34.89^{***}
012 526 612	1	13	13	5	0	5.53^{**}
011 113 094	3	7	15	1	0	1.27
015 316 612	2	32	0	3	0	-
011 895 051	1	9	0	0	0	-
013 559 035	2	10	8	0	0	-
011 554 623	2	8	6	0	0	-
014 523 892	1	1	3	0	0	-

Table 3.3b. The sex ratio of pooled progenies from control crosses. The sex ratio was compared to 1:1 ratio using a χ^2. n.s.: not significant.

	Female	Male	χ^2
Pooled progenies from control crosses	32	44	1.89 n.s.

wild type meiogynes died during the rearing period probably due to poor management. It should be noted that males were only observed when the pooled number of progenies exceeded 23. Contingency tests of male and female distribution between pooled progenies from each female showed homogeneity (χ^2= 11.637, d.f=8).

3.3.3. Gene-centromere recombination rate at ADA* locus in red meiotic gynogenetic progeny

The distribution of genotypes in meiotic gynogenetic progeny derived from 5 females heterozygous at *ADA** locus are presented in Table 3.4. The high recombination value (y=1) observed for the *ADA** locus in a total of 366 meiotic gynogenetic progeny from 5 heterozygous females suggests that a single crossover occurs between this gene and the centromere in all cases. The result also supports success in the production of meiotic gynogenetics. Fig. 3.3. A shows the *ADA** banding pattern of meiotic gynogenetic progeny derived from a heterozygous female and a homozygous male for the *ADA** locus. All control progeny have one of two paternal alleles from their heterozygous father but the other allele came from either of the two maternal alleles from their heterozygous mother making them homozygous or heterozygous at the *ADA** locus (Fig 3.3.B).

Table 3.4. Distribution of genotypes and proportion of heterozygous (Y) at *ADA** loci in meiotic gynogenetic progeny derived from heterozygous (at same loci) female parent of red tilapia, *O. niloticus*.

Female tag no.	Female parent genotype	Progeny phenotype			Proportion of heterozygous
		F/F	F/S	S/S	(y)
011 620 585	113/135	0	279	0	1
015 316 612	113/135	0	35	0	1
012 526 884	113/135	0	31	0	1
011 554 623	113/135	0	17	0	1
014 523 892	113/135	0	4	0	1

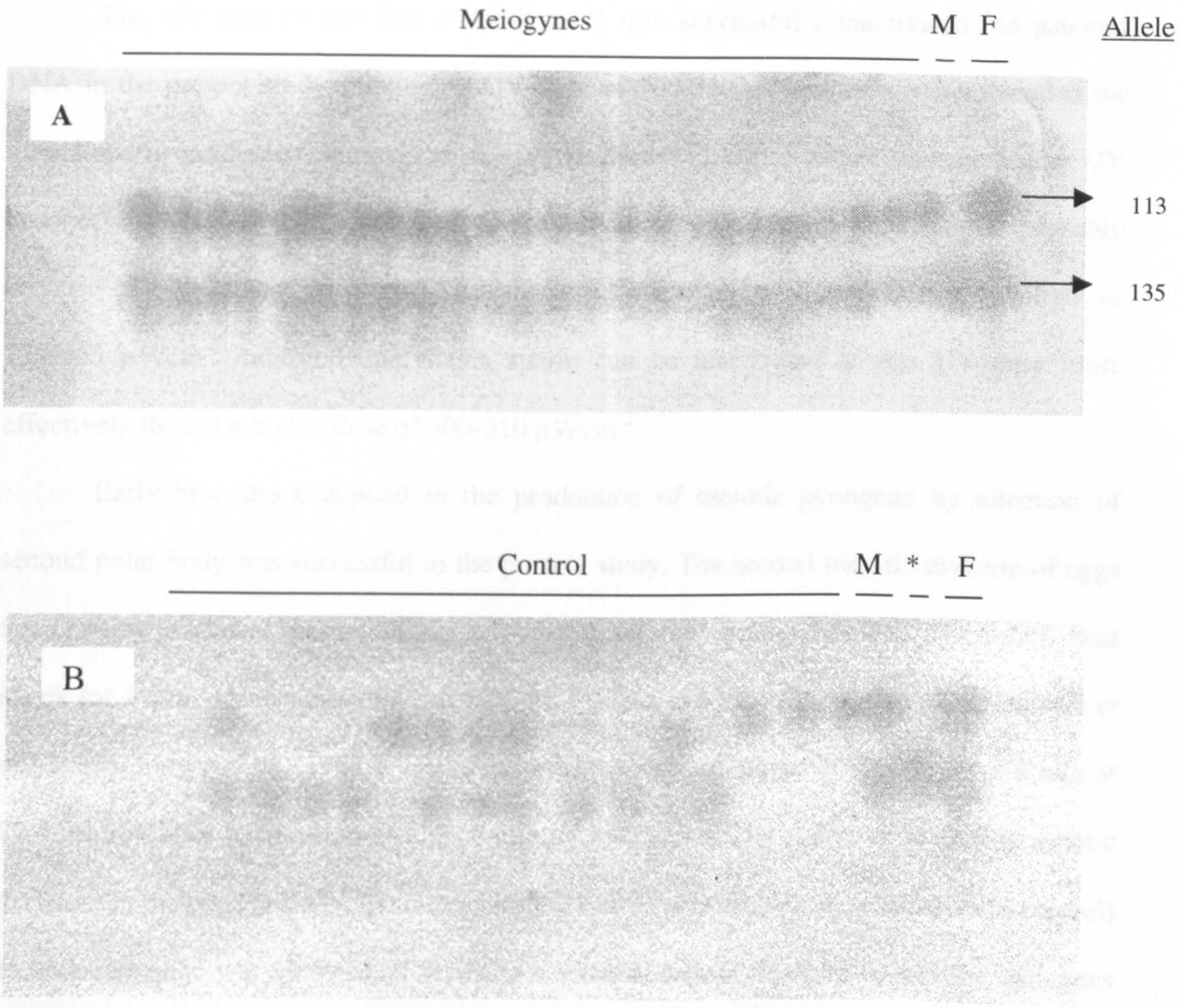


Fig. 3.3. Starch gel showing the *ADA** banding pattern of A) diploid meiotic gynogenetic B) control progeny groups derived from a heterozygous red female and male of *O. niloticus* (*: unrelated fish).

3.4. Discussion

The UV dose of 250-265 μWcm^{-2} for 2 min successfully inactivated the paternal DNA in the present study, although a UV dose of 300-310 μWcm^{-2} was recommended for tilapia sperm irradiation (Hussain *et al.*, 1993). Sarder (1998) reported that the higher UV dose of 300-310 μWcm^{-2} produced low survival of gynogenetic Nile tilapia, probably because of UV damage to sperm. The successful inactivation of sperm using UV light of 250-265 μWcm^{-2} indicated that tilapia sperm can be inactivated at this UV dose more effectively than at a higher dose of 300-310 μWcm^{-2}.

Early heat shock applied in the production of meiotic gynogens by retention of second polar body was successful in the present study. The second meiotic division of eggs fertilized by irradiated sperm was suppressed at 5 min post fertilization using 41.5-42°C heat shock for 4 min, which was similar to 41°C for 3.5 min at 5 min after fertilization (Hussain *et al.*, 1993). Myers *et al.* (1995a) used similar heat shock treatment of 42.5°C for 3-4 min at 22.5-30 min after fertilisation to induce mitotic gynogenesis by suppression of first mitotic division. In the present study, the mean survival rate of meiotic gynogens (relative to control) to yolk-sac stage was 19.59±3.58 % while a survival rate of 48±7.35 % meiotic gynogens (relative to control) was reported by Sarder (1998) using the same induction parameters in Nile tilapia. It seems likely that high survival of meiotic gynogens depends on the survival rate of the respective control group. In this respect, the mean survival rate of 33.09±3.08% in the control group in the present study is much lower than the mean survival rate of 63.11±6.41 % to yolk-sac stage reported by Sarder (1998).

According to Nace *et al.* (1970), Thorgaard *et al.* (1983) and Streisinger *et al.* (1986), the frequency of heterozygotes is expected to vary depending on the frequency of crossing

over between the gene and its centromere. No heterozygotes are expected if no crossing over takes place. All heterozygotes are expected if only one crossover always takes place and about 67 % heterozygotes are expected if the gene is assorting independently in relation to the centromere. The recombination frequencies of the red skin colour gene in meiotic gynogens produced from heterozygous red females (Rr) ranged between 0-0.447 with a mean of 0.118±0.04. Hussain *et al.* (1994) reported recombination rate varying between 0.2-1 with a mean of 0.45 at the same locus in meiotic gynogens derived from sibling heterozygous red female Nile tilapia and suggested that this gene could be situated near a recombination hotspot. It should be noted that the wide range of recombination frequencies obtained by Hussain *et al.* (1994) was derived from 6 gynogenetic experiment involving 6 sibling heterozygous red females and the distribution of red and wild type meiogynes produced showed significant heterogeneity (χ^2 = 16.54, degree of freedom 5, P<0.01) compared to the present study. No such heterogeneity was shown in the present larger study. In this respect, the present study finds no evidence for the recombination hotspot proposed by Hussain *et al.* (1994).

The results for the *ADA** locus suggest that exactly one crossover takes place between this gene and its centromere under complete interference where the occurrence of one crossover completely suppressed the occurrence of another. Therefore, the *ADA** locus should be placed distally from the centromere on its respective chromosome. High frequencies of gene-centromere recombinants have been observed in plaice (Thompson *et al.*, 1981; Thompson, 1983), rainbow trout (Thorgaard *et al.*, 1983; Guyomard, 1984; Thompson and Scott, 1984; Allendorf *et al.*, 1986), medaka (Naruse *et al.*, 1988), Nile tilapia (Hussain *et al.* 1994) and a mollusc (Guo and Allen, 1996). More recently, Kocher *et al.*

(1998) constructed a genetic map for Nile tilapia using DNA markers (62 microsatellite and 112 anonymous fragment length polymorphism) and observed high levels of interference over relatively large map distances. The authors suggested that high levels of interference suppressing a random recombination event on a chromosome increases the proportion of pairs that appear to be closely linked and thus reduces the estimate of genome size derived from closely spaced markers, and also high levels of interference reduces the proportion of pairs linked at longer distances, thus resulting in an increased genome size estimation.

The presence of a relatively high number of males (11%) in meiotic gynogens of tilapia in the present study was not very surprising, although it is not predicted by the homozygous female and heterozygous male system of sex determination in *O. niloticus* suggested by Penman *et al.* (1987), Mair *et al.* (1991a) and Carrasco *et al.* (1999). The occurrence of males among meiotic gynogenetic progeny could have arisen from an epistatic locus with a recessive allele causing female to male sex-reversal in the homozygous phase. It can be assumed that a female heterozygous for this locus (XXSRsr) could produce non-recombinant homozygous male (XXsrsr) and female (XXSRSR) and recombinant heterozygous female (XXSRsr). Mair *et al.* (1991a) observed 4.1% males in meiogynes and 20% male in mitogynes of *O. niloticus* and proposed rare recessive alleles at autosomal genes, inducing natural sex reversal from homozygosity of these alleles. Muller-Belecke and Hörstgen-Schwark (1995) reported 35.5 % male mitotic gynogens in *O. niloticus*. Hussain *et al.* (1994) obtained 7.5 % males in meiogynes and 47.5 % males in mitogynes in *O. niloticus* from the same batch of eggs and they also suggested an epistatic sex determining locus (*SDL-2*) with two alleles (SR and sr) which induce sex reversal from female to male in the homozygous recessive state as in the present study. More recently Sarder *et al.* (in press)

reported 25 % of males in the production of a clonal inbred line from a mitotic gynogenetic female and >60% males during further propagation of this line in *O. niloticus*. The authors suggested that the mitotic gynogenetic female might be homozygous for a recessive allele at an autosomal sex determining locus and fixation of some alleles or combination of alleles at different loci may cause sex reversal from female to male, but with partial penetrance since only a low percentage of males (25 %) was obtained instead of the expected 100 % males. In common carp, similar sex reversal from female to male was reported by Komen *et al.* (1992b) in production of clonal inbred lines by gynogenesis. They proposed an autosomal recessive sex determining gene, masculinization (*mas-1*), which produces males in the homozygous state (XX *mas-1/mas-1*) and females and intersexes in the heterozygous state (XX *mas-1/mas+1*).

A close linkage was found between a sex determining locus (called *SDL-2* with two alleles SR and sr) and the red locus in this study. A female which is heterozygous for a particular locus produces two types of meiotic tetrad: the sister chromatids are homozygous for the recessive allele or homozygous for the dominant allele or the sister chromatids are heterozygous because of recombination between gene and centromere. In the case of linked genes, the sister chromatids are either homozygous or heterozygous for dominant and recessive alleles. The possible half tetrad configuration of the females, heterozygous and linked for red (Rr) and sex determining locus (SRsr) is presented in Fig. 3.4. According to the half tetrad configuration in Fig.3.4 A., the females would be expected to produce equal number of non-recombinant homozygous red females (RRSRSR) and homozygous wild type males (rrsrsr) while recombinant phenotype would be heterozygous red female (RrSRsr). However, all the meiogyne males produced in the present study were red except one wild

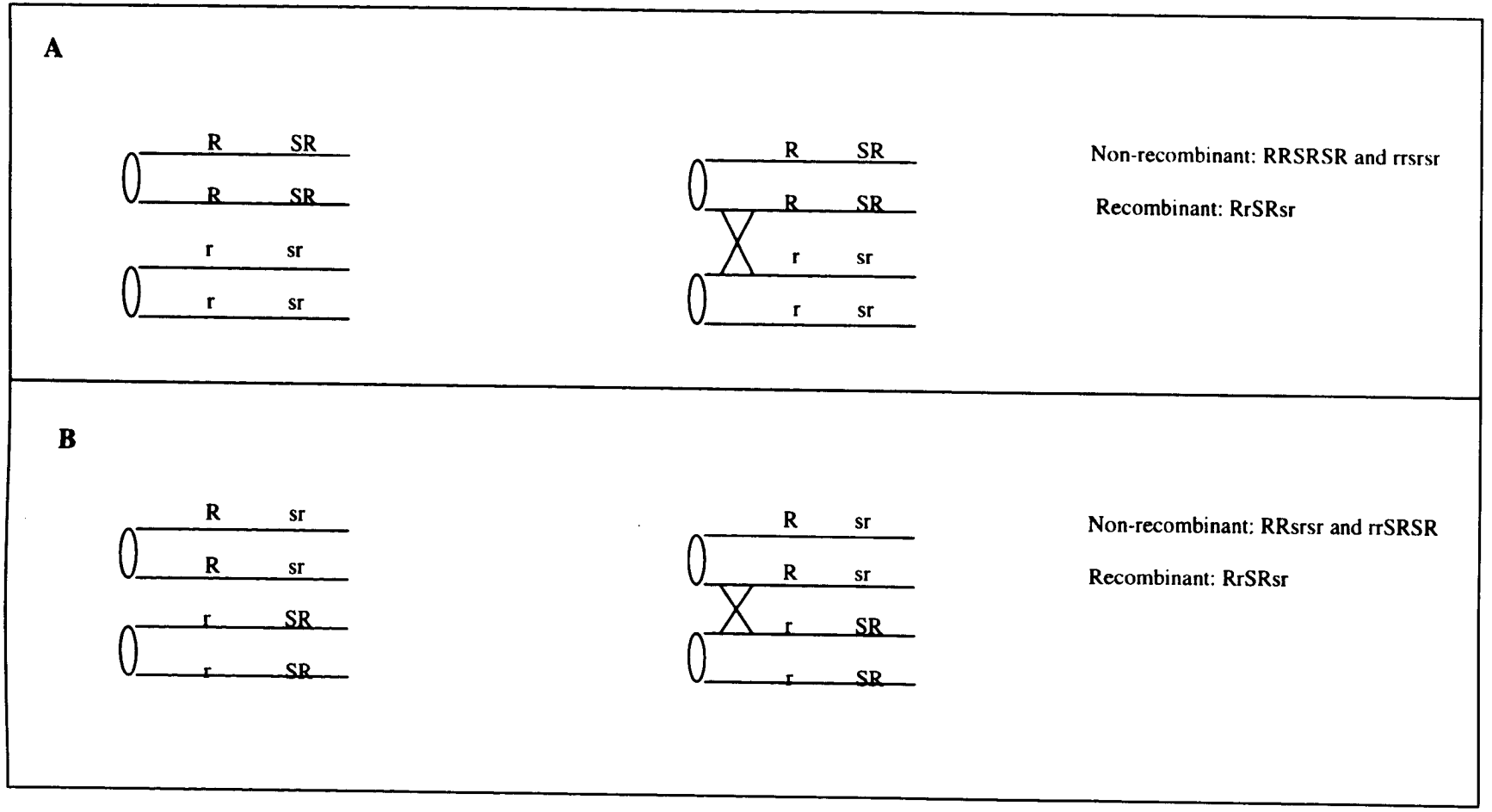


Figure 3.4. Schematic diagram of possible half tetrad configurations (A and B) of prophase I in meiotic gynogenesis between red and sex determining locus, SDL-2.

type male derived from the female 011 620 585 suggesting that this half tetrad configuration of the females is not valid for explanation of the occurrence of red males in gynogenetic experiments. On the other hand, according to the alternative half tetrad configuration of females shown in Fig.3.4.B., the females would be expected to produce equal number of non-recombinant homozygous red males (RRsrsr) and homozygous wild type females (rrSRSR) and recombinant heterozygous red females (RrSRsr). In this case, the number of pooled non-recombinant homozygous wild type meiotic female progenies (n=230, rr SRSR) should be equal to the number of pooled non-recombinant homozygous red meiotic male progenies (n=230, RRSRSR) whereas the remaining heterozygous red meiotic females (n=86, RrSRsr) should be the recombinant for red and wild type alleles of both linked genes. Wild type meiotic progeny were 100 % female with an exception of one wild type meiotic male from the female 011 620 585. On the other hand, the estimated frequency of males in the non-recombinant red progeny was 23.04 %. The result of the present study cannot be explained with the suggested model above unless partial penetrance of this recessive sex determining allele in the homozygous condition (srsr) which causes female to male sex reversal considered. In this respect, the present study agreed with Sarder *et al.* (in press) with a 23.04 % penetrance frequency, which is quite similar to 25 % maleness in their inbred clone.

The occurrence of the single unexpected wild type gynogenetic male was not predicted from the suggested model above. The occurrence of this wild type gynogenetic male could have been arisen from rare natural sex reversal event. Rare spontaneous sex reversal was also reported by Scott *et al.* (1989) and Mair *et al.* (1991a) in *O. niloticus*. Occurrence of intersex *O. niloticus* during sex reversal treatment (Rothbard *et al.*, 1981;

Mair *et al.*, 1987b) and crosses between fish of normal (Mair *et al.*, 1991a) could be indicative of spontaneous sex reversal. The wild type male may also result from the normal fertilisation of the sperm which escape from the irradiation.

Chapter 4:

The effect of colouration on reproductive traits of strip-spawned *O. niloticus* over consecutive spawns

4. The effect of colouration on reproductive traits of strip-spawned *O. niloticus* over consecutive spawns

4.1. Introduction

The low fecundity and asynchronous spawning patterns of female tilapia lead to the need for extensive holding facilities and the management of relatively large numbers of broodstock compared to most other commercial fish species. Therefore, broodfish productivity of any tilapia species is one of the most significant constraints on commercial production costs, and knowledge of the factors affecting seed production are crucial. These include broodstock nutrition, husbandry conditions, genotype of the broodfish and pleiotropic effects of any gene that might influence seed production in tilapia culture.

4.1.1. Reproductive biology of tilapias: Reproduction modes and traits

Members of the genus *Oreochromis* adopt a maternal mouthbrooding mode of reproduction. Male fish build and defend the territories within a defined spawning area, called a "lek". A female ready to spawn visits the lek to have the eggs fertilized by one or more of the nest-holding males (Wootton, 1990). Courtship is relatively short, lasting just a few hours (Rana, 1988) and results in batches of eggs being spawned into shallow nests. Fertilized eggs are picked up by the female and incubated in her mouth until the fry are free-swimming (Rana, 1988).

Fecundity in tilapias has been defined in various ways. Lowe-McConnell (1955: cited in Rana, 1988) defined fecundity as the numbers of fry produced in the lifetime of

an individual fish. However, broodstock would normally only be used during their optimum reproductive period under culture conditions (Rana, 1988). As a result, Mires (1982) and Macintosh (1985) suggested that the definition should be restricted to the number of fry produced over a 12 month period. The number of maturing oocytes present in the ovaries of females prior to spawning is also accepted as another measure of fecundity in fish (Bagenal, 1978).

In multiple spawners like tilapias, however, the recruitment of oocytes for maturation appears to be more complex since very ripe ovaries show a bimodal oocyte size distribution (Peters, 1983; Srisakultiew, 1993; Coward, 1997). This is less marked at other stages of the ovarian cycle. Considering the uncertainty of which oocytes will contribute to the next spawning (Jalabert and Zohar, 1982) and the presence of atretic eggs in the ovary which shows not all are spawned (Peters, 1983; Rana, 1986), this might lead to an overestimation of total fecundity and its relationship with body size. Therefore, the fecundity of tilapias is best defined as the number of eggs in a freshly spawned clutch (Rana, 1988) and the present study adopts this definition.

In tilapias, the evolution of parental care has led to a resultant increase in egg size and a corresponding reduction in the number eggs per clutch (Fryer and Iles, 1972, Peters, 1983; Trewavas, 1983). *O. niloticus* females produce relatively few large eggs in each spawning. Although their egg numbers are small, hatching rates of eggs and survival of fry are high (Peters, 1983; Rana, 1988). In tilapia species, egg size is reported to be species-specific (Lowe-McConnel, 1955: cited in Rana, 1988; Trewavas, 1983), larger eggs generally produced by bigger individuals within a species. It is generally accepted in teleost fish that total fecundity increases and relative fecundity (number of eggs/unit body

weight) decreases in accordance with increasing female age, length and weight (Wootton, 1990). This relationship has also been reported for tilapia in which larger eggs are produced from larger females (Siraj *et al.*, 1983; Trewavas, 1983; Rana, 1986). Rana (1986), however, reported that, in *O. niloticus* total fecundity is more closely related to maternal size than age and fecundity of similar aged fish increased with body size.

Spawning frequencies of multiple-spawning individual tilapia broodstock exhibit great variability according to several factors such as fish size, temperature, latitude, degree of paternal care, stocking density, sex ratio, food ration and dietary protein level (Wootton, 1990). Peters (1983) reported that wild tilapias spawned at least twice a year. At high altitudes, tilapia have only a 3-4 month spawning period during which only three spawning cycles can be expected (Mires, 1982). Phillipart and Ruwet (1982) reported that spawning cycles of tilapias might occur monthly in the tropics. On the other hand Fishelson (1966) using captive tilapias maintained under controlled environmental conditions found no evidence of seasonality and obtained 11 clutches of eggs in a year from a single female robbed of her clutch after spawning. Mires (1982) reported that *O. niloticus* females spawned only 2-7 times/year at average breeding cycles of 23-50 days under controlled spawning conditions. Hughes and Behrends (1983) suggested that stocking density and sex ratio of broodfish might alter the spawning frequencies. The selected sex ratio should be sufficient for a female to easily find a male with which to spawn (Little, 1989).

Little is known about the effect of successive spawning pattern on reproductive biology of tilapia species. Studies on *O. esculentus* suggested that the gonads may be larger and produce more eggs per spawn towards to the end of the spawning period

(Lowe-McConnell, 1955: cited in Rana, 1988). Siraj *et al.* (1983), studying three year classes of *O. niloticus* over 3 spawning periods length found that relative fecundity decreased with successive spawning periods. Srisakultiew (1993) found that fecundity of a single *O. niloticus* female was higher at the second and third spawning than first spawning cycle.

Several methods have been used in attempting to enhance the egg and fry production in various tilapiine fishes, hypophysation and temperature manipulation (Srisakultiew and Wee, 1988), removal of eggs from the buccal cavity (egg/fry robbing) of mouthbrooding species, stocking broodstock at optimal sex ratio, density (Siddiqui and Al-Harbi, 1995a; Bautista *et al.*, 1988) and age (Ridha and Cruz, 1989) and varying nutrient supply (Cisse, 1988).

4.1.2. Pleiotropy

Pleiotropy was defined as: the production of additional or secondary phenotypes that occur because a gene influences two or more phenotypes by Tave (1993). Although pleitropic effects seems insignificant if no economically important characteristics are altered, it can become important if any economically important characteristics such as viability, productivity and market value are positively or negatively affected (Tave, 1993).

Negative pleitropic effects of both blue (bb) and gold (gg) common carp on growth rates were reported by Wohlfarth and Moav (1970: cited in Tave, 1993). On the other hand Wlodek (1968: cited in Tave, 1993) reported that Polish blue common carp grew better than normally pigmented common carp.

Pleiotropic effects of scale patterns (mirror, line and leather) on common carp have been extensively studied and 17 pleiotropic effects on traits such as weight, mean number of soft rays in dorsal, anal and pelvic fin, ability to regenerate fins and survival have been detected (Tave, 1993).

The S allele producing saddlebacks in heterozygous state (S+) caused vertebral anomalities in several vertebrae, decreased disease resistance and reduced viability in *O. aureus* (Tave, 1993).

The negative pleiotropic effects of "a" allele in channel catfish causing albinism in the homozygous state (aa) on egg size, egg quality, hatching rate, viability and growth rate have been reported by Bondari (1984). On the other hand, Kincaid (1975: cited in Tave, 1993) reported that "b" allele producing iridescent metallic blue body colour in rainbow trout improved growth rate.

Negative pleiotropic effect of the red phenotype in *O. aureus* X *O. niloticus* hybrid on survival rate during early stages of development and pond culture due to differential predation by birds and fish was reported by Behrend *et al.* (1982) and El-Gamal *et al.* (1988).

4.1.3. Objectives

The evidence from other fish species shows that the viability and reproductive traits of *O. niloticus* could be affected by its colour. Thus the present study was aimed to investigate and compare several reproductive traits in three colour genotypes namely homozygous red (RR), heterozygous red (Rr) and homozygous wild type (rr) in laboratory held stocks of *O. niloticus* in order to examine potential pleiotropic effects.

The colour variants were studied over successive reproductive cycles using highly reproducible techniques standardized for the all year round breeding conditions for *O. niloticus* used in the Institute of Aquaculture. The pleiotropic effects of red genotype on the survival rates of *O. niloticus* at different developmental stages were also taken into consideration.

4.2. Materials and Methods

4.2.1. Experimental fish, stocking facilities and fish maintenance

The three colour genotypes of *O. niloticus* namely homozygous red (RR), heterozygous red (Rr) and homozygous wild type (rr), showing three distinct colour morphs of pure red, red with black blotching and wild type (Plate 1.1. and 1.2.) were obtained from genetically pure broodstock held at the Institute of Aquaculture, University of Stirling, Scotland (McAndrew and Majumdar, 1983a; McAndrew *et al.*, 1988).

All the fish used in this study were maintained according to Section 2.2.1. The fish were fed *ad libitum* twice daily with a commercial pelleted trout feed (Trouw Aquaculture Nutrition, Russhive, U.K.). All procedures requiring fish handling e.g. stripping, tagging and weighing were performed under anaesthesia (Section 2.2.2). All broodstock were individually PIT-tagged (Section 2.2.3).

4.2.2. Stripping, fertilising and incubating of eggs

Stripping of eggs was performed according to Section 2.2.4. After stripping, fish were blot dried on absorbent tissue paper and weighed (to the nearest 0.1 g) on a Mettler 400 balance (Fisons Scientific Equipment, U.K.) and the standard length determined (to the nearest 0.1 mm) using a scaled ruler fitted to a wooden frame.

After egg collection, fertilisation was carried out *in vitro* using "dry milt" (Section 2.2.4). Fertilised eggs were incubated according to Section 2.2.4.1. Survival rates were checked at pigmentation, hatching and yolk sac stages (Section 2.2.9).

4.2.3. Determination of total fecundity, reproductive parameters and estimation of spawning frequency

4.2.3.1. Determination of total fecundity

As discussed in Section 4.2, there is a considerable debate in the literature over the precise definition of fecundity in the multiple spawning tilapiine family of fish. In this present study, the definition of Rana (1986) who stated that total fecundity of tilapias should only be defined as the number of discrete eggs found in a freshly spawned egg clutch was used. All measurements of fecundity and egg size in this study were made on water hardened eggs from individually held female fish artificially stripped under anaesthesia. The eggs were counted manually using a fine paintbrush. An egg clutch containing approximately 1000 eggs could be counted within 10 minutes.

4.2.3.2. Reproductive parameters

Since the eggs of mouth brooding tilapia are ovoid-shaped, two parameters were measured in calculating mean egg diameter and mean egg volume, egg long axis referring to the length of the longest axis of the egg, and egg short axis referring to longest width at right angle the long axis.

A sub-sample of each batch of eggs was placed in a Petri dish with water and then photocopied (Model 5053, Xerox Ltd. UK). The images were scanned by using a scanner (Epson GT-9500) and long and short axes of 40 randomly chosen eggs from 5 females of homozygous red (RR), heterozygous red (Rr) and homozygous wild type (rr) *O. niloticus* were measured using a computer software package (Image Pro Plus Version 3.0). Mean

egg diameter and mean egg volume were calculated using equations described by Coward and Bromage (1999).

Determination of mean egg diameter

The mean egg diameter was calculated using the following equation from individual eggs;

$$D = (l+s)/2$$

where:

D= egg diameter (mm)

l= length of egg long axis (mm)

s= length of egg short axis (mm)

Determination of mean individual egg volume

The mean volume of individual eggs was calculated using the following equation;

$$V=(\Pi/6)*l*h^2$$

where:

V= volume of egg (mm^3)

l= length of egg long axis (mm)

h= length of egg short axis (mm)

Determination of mean individual egg weight

A sub-sample of 500 eggs were placed in a small net and excess water was removed by blotting with a tissue. The eggs then placed in a pre-weighed Petri dish and weighed to the nearest ≥0.1g a on an electronic balance (Mettler AA 100).

Determination of relative fecundity

Relative fecundity (expressed as eggs/g body weight) was calculated using the following equation;

$$\mathbf{RF = TF/W}$$

where:

RF= relative fecundity (eggs/g)

TF= total fecundity (no of eggs in clutch)

W= weight of fish (g)

Determination of total egg volume

Total egg volume (expressed as mm^3) was calculated using the following equation;

$$\mathbf{TEV = TF*MEV}$$

Where:

TEV= total egg volume (mm^3)

TF= total fecundity (no of eggs in clutch)

MEV= mean egg volume (mm^3)

Determination of EW: BW ratio

Egg weight to body weight ratio (EW: BW, expressed as %) was calculated using the following equation;

$$\mathbf{EW:BW = (EWW*TF/W)*100}$$

Where:

EW:BW= egg weight to body weight ratio (%)

EWW= egg wet weight (mg)

TF= total fecundity (no of eggs in clutch)

W= weight of the fish

Estimates inter-spawning-interval (ISI)

ISI (inter-spawn-interval) is based on completed reproductive cycles of repeat spawning fish, i.e. the time elapsed from one spawn to the next.

4.2.4. Experimental design

In total 10 homozygous red (RR), 14 heterozygous red (Rr) and 10 homozygous wild type (rr) randomly chosen *O. niloticus* brood fish were observed and strip-spawned in this study. Although the spawning history of these brood fish was unknown, they were all 2+ years old at the beginning of the study and had been kept under standard conditions.

Each female was checked daily for ovulation (at approximately 1 p.m.) and ovulated females were artificially stripped (between 2-4 p.m.), weighed, measured (Section 4.2.2) and the eggs were counted (Section 4.2.3.1) for determination of fecundity and ISI. This was continued for 11 months starting from April, 1997 to March, 1998 except July, 1997.

For determination of egg size and weight, the eggs of 5 randomly selected females from each colour genotype were used once according to Section 4.2.3.2.

Several crosses were performed between different colour genotypes of *O. niloticus* in order to determine the effect of colour genotype on the survival of *O. niloticus*. For this purpose, several homozygous red and wild type female tilapia were artificially stripped and the eggs were divided into two groups. One of the groups was

fertilized with "dry" milt from a homozygous red male and the other with milt from a wild type male tilapia. The survival rate of each group was determined according to Section 4.2.2.

4.2.5. Statistical analyses

The normality of the data was tested by the Anderson-Darling Normality test. Monthly comparisons of reproductive traits in terms of length, weight, total fecundity and relative fecundity of each colour genotype were tested by one-way ANOVA followed by a Tukey test in normally distributed data. Non-normally distributed data were analysed by the Kruskal-Wallis Test (Sokal and Rohlf, 1987; Gardiner, 1997).

Comparisons of reproductive traits in terms of total fecundity, ISI, egg weight, egg diameter, egg volume, EW:BW ratio and total egg volume were tested by analyses of covariance (ANCOVA) using the general linear model (GLM) (Ryan and Joiner, 1994). This techniques combines the statistical techniques of regression and ANOVA and tests a dependent variable "y" for homogeneity among colour genotype means similar to ANOVA. Before means are tested, they are adjusted for differences between colour genotypes in the independent variable "x" (the covariate). Since the same females were used for investigations of fecundity and ISI during the experimental period, female tag number was used as nested factors within each colour genotype for comparison of total fecundity and ISI between different colour genotypes. The main significance test involved in GLM is a test of homogeneity of residual variances. Therefore, the homogeneity of residuals was tested by the Anderson-Darling Normality test. Normally distributed raw data of reproductive parameters (total fecundity, ISI, egg weight, egg

diameter, egg volume, EW:BW ratio and total egg volume) were used individually as dependent "y" variables and were tested against post-spawned fish weight and length as the independent "x" variables. Only the data of ISI as dependent "y" variable and total fecundity as independent variable "x" were $\log_{10}$ transformed for normality. The data for relative fecundity were not subjected to ANCOVA for comparison of colour genotypes because the number of eggs produced for each unit increase in weight shows considerable linear variation and attempts to correlate weight with relative fecundity are also subject to autocorrelation (Bromage *et al.*, 1990). However, relative fecundities are used by fish farmers to assess stock productivity (Bromage *et al.*, 1990). Therefore, these data were included in most cases.

After arcsine transformation of survival rates (%) of crosses involving different colour genotypes, one-way ANOVA and Tukey tests were performed to compare each developmental stage. The results were presented as mean and standard error of mean (±SE) without arcsine transformation. Female effect, male effect and female *male interaction were tested using general linear model since the experimental design was unbalanced (Ryan and Joiner, 1994).

Relationships between fish weight and length with total fecundity, relative fecundity, egg wet weight, egg diameter, egg volume, total egg volume and EW:BW were investigated using correlation and regression analyses on untransformed and $\log_{10}$ transformed data. All statistical analyses were performed using Minitab 9.2 software.

The coefficient of variation (C.V.) forms a measure of variability and was used to compare variation in terms of ISI, weight, length, total fecundity and relative fecundity of

individual fish from each colour genotype of *O. niloticus* over consecutive spawnings. The coefficient of variation is derived as follows;

%C.V.= (s*100) / x

where;

s = standard deviation of the data being tested

x = sample mean of the data being tested

4.3.Results

In total 10 homozygous red (RR), 14 heterozygous red (Rr) and 10 homozygous wild type (rr) tilapia of similar age were observed and strip-spawned over 11 months. Although there were no mortalities, several fish became injured due to fighting after the collapse of Perspex-partitions used to separate the glass aquaria. This allowed 2 previously segregated fish access to one another and usually led to the more dominant of the two fish injuring the other.

The initial weights of females of each colour genotype were not significantly different ($P>0.05$). Strip spawning was always performed between 2-6 p.m. depending on the condition of the urogenital papilla of females. All parameters (weight, length, total fecundity, etc) were measured on freshly stripped females. However, several natural spawnings also occurred in some females, which did not show any physical appearance of readiness. In this case only the date of spawning was recorded. Although counting of eggs was performed easily using a fine paintbrush on good eggs, in some clutches, the eggs were soft and burst easily making it impossible to count them. Therefore, in such cases, only weight, length and date of spawning could be recorded.

4.3.1. Monthly changes in reproductive traits of homozygous red (RR) O. niloticus over consecutive spawns

Monthly values of weight, length, spawning frequency and fecundity of 10 homozygous red (RR) *O. niloticus* are depicted in Table 4.1 and graphically presented in Fig. 4.1. At the beginning of the experiment the weight and length of fish ranged between 178.6-234.0 g (mean 205.9±16.0 g) and 18.2-19.6 cm (mean 18.8±0.4 cm), respectively.

The mean weight and length of fish had significantly increased by the end of the experimental period ($P<0.05$). All the fish spawned in month 4. (August), 5. (September) and 6. (October) while only 4 and 6 fish spawned in month 1. (April) and 11. (March) of the experimental period, respectively.

There were no significant differences in mean total fecundity and relative fecundity between months ($P>0.05$).

4.3.2. Monthly changes in reproductive traits of heterozygous red (Rr) *O. niloticus* over consecutive spawns

Monthly values of weight, length, spawning frequency and fecundity of 14 heterozygous red (Rr) *O. niloticus* are presented in Table 4.2. and Fig. 4.1. The mean weight of 214.2±11.3 g and length of 19.4±0.4 cm in first month were significantly different from the last month values of 310±11.8 g and 22.1±0.3 cm, respectively, showing the fish had grown over this period.

All fish spawned during month 4. (August) and 5. (September) while only 9 fish spawned in the last month (March) of experimental period.

Although, there were no significant differences between the months in total fecundity ($P>0.05$), significant differences were found between month 2. (May), 8. (December) and 9. (January) in relative fecundity ($P<0.05$).

Table 4.1. Monthly values of fish weight, length, and fecundity of homozygous red (RR) *O. niloticus* maintained in partitioned glass aquaria (based on spawned fish). Number of fish = 10; the same superscripts in the same column are not significantly different (P>0.05).

Month	Mean weight of fish (g±SE)	Mean length of fish (cm±SE)	No. of fish spawned once	No. of fish spawned more than once	Total no. of spawned fish	Mean fecundity (±SE)	Mean relative fecundity (eggs/g±SE)
1. (April)	205.9±16.0 ^{ab}	18.8±0.4^{a}	4	-	4	1058.7±100.6^{a}	5.14±0.3^{a}
2. (May)	228.6±19.3 ^{ab}	19.9±0.6^{ab}	3	4	7	1007.5±170.1^{a}	4.7±1.2^{a}
3. (June)	229.5±17.8 ^{ab}	20±0.5^{ab}	5	4	9	1181.4±116.3^{a}	5.3±.0.7^{a}
4. (August)	203.4±46.9^{a}	19.1±0.5^{a}	5	5	10	1055.2±76.4^{a}	5.7±0.4^{a}
5. (September)	255.4±15.2 ^{ab}	19.3±0.5^{ab}	5	5	10	1109.8±87.6^{a}	5.3±0.5^{a}
6. (October)	238.5±14.7 ^{ab}	20.0±0.4^{ab}	8	2	10	1198.9±54.2^{a}	5.2±0.4^{a}
7. (November)	227.1±11.2 ^{ab}	19.6±0.4^{ab}	5	2	7	1122.3±101.3^{a}	5.1±0.6^{a}
8. (December)	251.6±12.6 ^{abc}	19.9±0.4^{ab}	5	2	7	1227.0±90.4^{a}	5.5±0.4^{a}
9. (January)	261.5±20.1 ^{abc}	20.5±0.5^{ab}	4	1	5	1201.8±60.4^{a}	4.7±0.4^{a}
10. (February)	282.0±16.2 ^{bc}	20.9±0.4^{ab}	6	1	7	1102.3±118.1^{a}	4.0±0.5^{a}
11. (March)	326.2±21.1^{c}	21.8±0.6^{b}	5	1	6	1152.7±81.0^{a}	3.8±0.2^{a}

Table 4.2.Monthly values of fish weight, length, and fecundity of heterozygous red (Rr) *O. niloticus* maintained in partitioned glass aquaria (based on spawned fish). Number of fish = 14; the same superscripts in the same column are not significantly different (P>0.05).

Month	Mean weight of fish (g±SE)	Mean length of fish (cm±SE)	No. of fish spawned once	No. of fish spawned more than once	Total no. of spawned fish	Mean fecundity (±SE)	Mean relative fecundity (eggs/g±SE)
1. (April)	215.2±11.3^{a}	19.4±0.4^{a}	11	1	12	1092.0±104.8^{a}	5.1±0.5^{ab}
2. (May)	211.8±11.4^{a}	19.5±0.4^{ab}	7	2	9	1124.6±52.6^{a}	5.4±0.4^{b}
3. (June)	211.0±8.2^{a}	19.9±0.3^{ab}	10	3	13	981.9±77.8^{a}	4.8±0.5^{ab}
4. (August)	227.3±10.0^{a}	20.3±0.3^{ab}	9	5	14	1154.8±104.0^{a}	5.1±0.4^{ab}
5. (September)	254.3±11.4^{ab}	21.0±0.3^{bc}	10	4	14	1194.7±23.7^{a}	4.8±0.3^{ab}
6. (October)	261.8±9.7^{ab}	21.1±0.3^{c}	11	-	11	1133.3±91.9^{a}	4.3±0.4^{ab}
7. (November)	293.4±14.2^{b}	21.7±0.4^{c}	8	3	11	1097.2±73.3^{a}	3.8±0.3^{ab}
8. (December)	306.6±14.1^{b}	22.2±0.3^{c}	8	-	8	1083.3±79.3^{a}	3.5±0.3^{a}
9. (January)	290.3±16.6^{b}	21.7±0.4^{c}	9	3	12	1060.2±90.0^{a}	3.7±0.5^{a}
10. (February)	302.1±13.0^{b}	21.8±0.3^{c}	8	1	9	1177.5±37.6^{a}	4.0±0.3^{ab}
11. (March)	310.1±11.8^{b}	22.1±0.3^{c}	8	1	9	1375.3±47.8^{a}	4.6±0.4^{ab}

4.3.3. Monthly changes in reproductive traits of homozygous wild type (rr) *O. niloticus* over consecutive spawns

Monthly values of fish weight, length, spawning frequency and fecundity of 10 homozygous wild type (rr) *O. niloticus* are shown in Table 4.3 and graphically presented in Fig 4.1. The mean fish weight of 248.3±16.1 and length of 20.0±0.4 cm increased significantly from the beginning of the experiment period to the last month (March) of the experiment period ($P<0.05$) and reached to maximum weight of 349.3±12.8 g and length of 22.7±0.3 cm.

Although there were no significant differences between months in terms of relative fecundity ($P>0.05$), the total fecundity was significantly ($P<0.05$) different between month 3. (June) (884.5±116.6) and month 5. (September) (1293.6±106.1).

4.3.4. Comparison and linear regression analysis of reproductive traits of homozygous red (RR), heterozygous red (Rr) and homozygous wild type (rr) *O. niloticus*

Table 4.4 shows the comparison between the three different genotypes of *O. niloticus* in terms of total fecundity, ISI, egg weight, mean egg diameter, mean egg volume, EW:BW ratio and total egg volume using analyses of covariance (ANCOVA). No significant differences were found between different colour genotypes of *O. niloticus* in terms of the reproductive traits mentioned above ($P>0.05$). However, the ANCOVA may not have been robust for mean egg diameter and ISI using fish weight and length as covariates since the residuals of variances were found to be heterogeneous for these traits.

Table 4.3. Monthly values of fish weight, length, and fecundity of homozygous wild type (rr) *O. niloticus* maintained in partitioned glass aquaria (based on spawned fish). Number of fish = 10; the same superscripts in the same column are not significantly different (P>0.05).

Month	Mean weight of fish (g±SE)	Mean length of fish (cm±SE)	No. of fish spawned once	No. of fish spawned more than once	Total no. of spawned fish	Mean fecundity (±SE)	Mean relative fecundity (eggs/g±SE)
1. (April)	248.3±16.1^{a}	20.0±0.4^{a}	5	3	8	1067.8±96.0^{ab}	4.4±0.4^{a}
2. (May)	253.0±12.3^{ab}	20.4±0.3^{a}	5	5	10	955.3±77.1^{ab}	3.8±0.3^{a}
3. (June)	245.4±12.0^{a}	20.1±0.3^{a}	7	-	7	884.5±116.6^{a}	3.6±0.5^{a}
4. (August)	267.4±16.8^{a}	21.0±0.5^{ab}	7	2	9	950.9±57.13^{ab}	3.6±0.3^{a}
5. (September)	299.0±12.6^{ab}	21.7±0.2^{b}	7	1	8	1293.6±106.1^{b}	4.6±0.4^{a}
6. (October)	300.3±15.0^{ab}	21.9±0.4^{b}	7	1	8	1103.9±83.2^{ab}	3.8±0.3^{a}
7. (November)	293.0±9.6^{ab}	21.6±0.2^{ab}	6	-	6	1056.8±54.6^{ab}	3.7±0.3^{a}
8. (December)	312.2±10.2^{bc}	21.9±0.2^{b}	4	4	8	1075.9±88.1^{ab}	3.5±0.3^{a}
9. (January)	308.6±8.15^{bc}	22.0±0.3^{b}	7	2	9	1010.2±94.3^{ab}	3.3±0.3^{a}
10. (February)	335.9±15.6^{c}	22.4±0.3^{b}	7	-	7	1162.3±95.5^{ab}	3.5±0.2^{a}
11. (March)	349.3±12.8^{c}	22.7±0.3^{b}	4	2	6	1147.4±56.0^{ab}	3.4±0.2^{a}

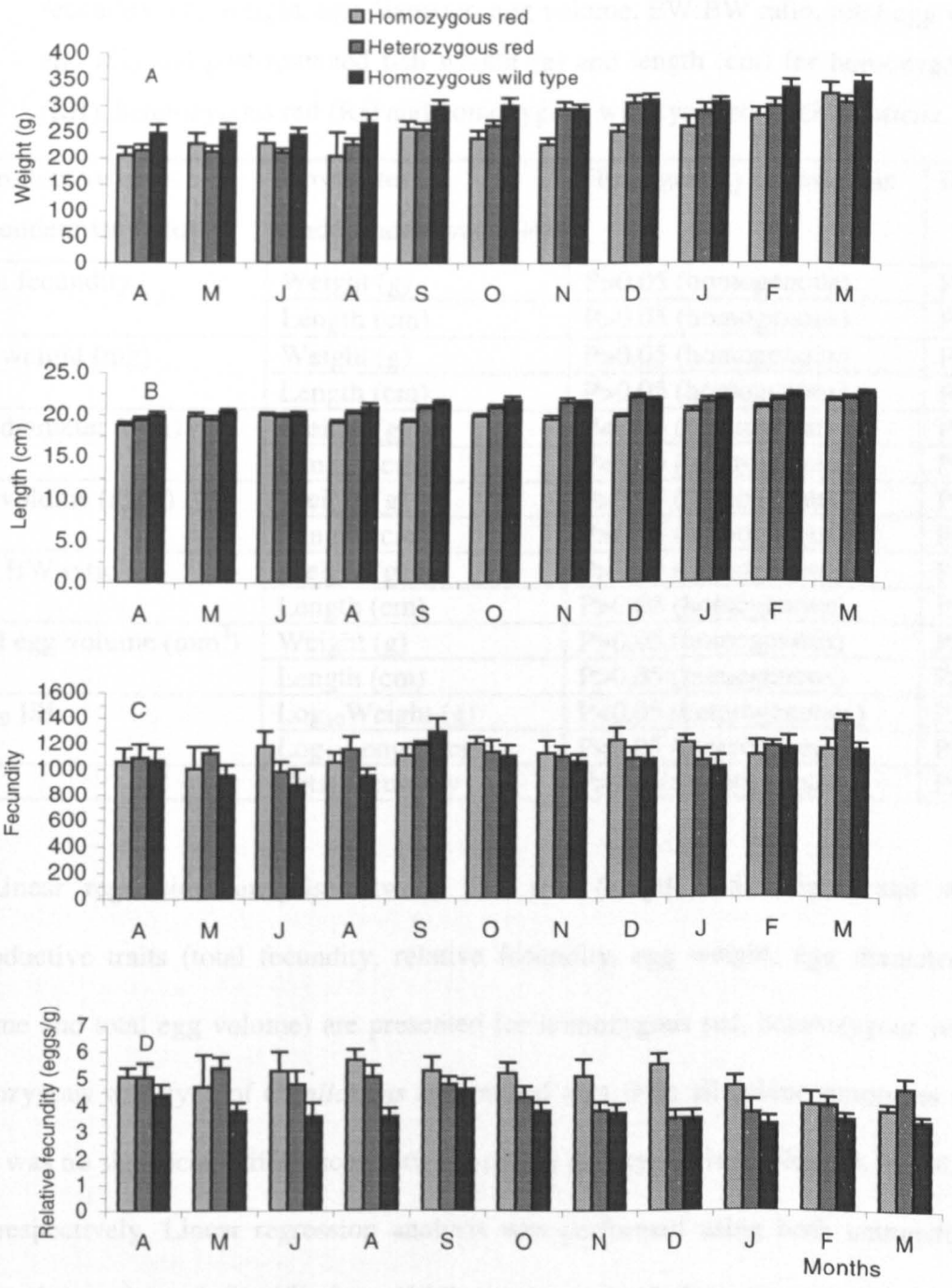


Figure 4.1. Mean monthly values of fish weight (A), length (B), fecundity (C) and relative fecundity (D) for homozygous red, heterozygous red and homozygous wild type *O. niloticus* (based on spawned fishes).

Table 4.4. Analyses of covariance (ANCOVA) of several reproductive traits (total fecundity, egg weight, egg diameter, egg volume, EW:BW ratio, total egg volume and ISI) and post-spawned fish weight (g) and length (cm) for homozygous red (RR), heterozygous red (Rr) and homozygous wild type (rr) of *O. niloticus*.

Reproductive traits (dependent variable)	Covariates (independent variable)	Homogeneity of residuals	Results
Total fecundity	Weight (g)	P>0.05 (homogenous)	P>0.05
	Length (cm)	P>0.05 (homogenous)	P>0.05
Egg weight (mg)	Weight (g)	P>0.05 (homogenous)	P>0.05
	Length (cm)	P>0.05 (homogenous)	P>0.05
Egg diameter (mm)	Weight (g)	P<0.05 (heterogeneous)	P>0.05
	Length (cm)	P<0.05 (heterogeneous)	P>0.05
Egg volume (mm^3)	Weight (g)	P>0.05 (homogenous)	P>0.05
	Length (cm)	P>0.05 (homogenous)	P>0.05
EW: BW ratio	Weight (g)	P>0.05 (homogenous)	P>0.05
	Length (cm)	P>0.05 (homogenous)	P>0.05
Total egg volume (mm^3)	Weight (g)	P>0.05 (homogenous)	P>0.05
	Length (cm)	P>0.05 (homogenous)	P>0.05
Log_{10} ISI	Log_{10}Weight (g)	P<0.05 (heterogeneous)	P>0.05
	Log_{10}Length (cm)	P<0.05 (heterogeneous)	P>0.05
ISI	Total fecundity	P>0.05 (homogenous)	P>0.05

Linear regression analysis between fish size (length and weight) and several reproductive traits (total fecundity, relative fecundity, egg weight, egg diameter, egg volume and total egg volume) are presented for homozygous red, heterozygous red and homozygous wild type of *O. niloticus* and pooled data from all colour genotypes (since there was no significant differences between colour genotypes) in Tables 4.5, 4.6, 4.7 and 4.8, respectively. Linear regression analysis was performed using both untransformed data and transformed (log10) data. While untransformed data are more immediately comprehensible, the recommended procedure for this type of data analysis involves logarithmic transformation (Sokal and Rohlf, 1987). Therefore, only the results of those analyses involving transformed data are described here.

1) Total fecundity (Fig.4.2.): Significant positive relationships were found between total fecundity and length in heterozygous red females ($P<0.05$), homozygous wild type ($P<0.01$) and pooled data from all colour genotypes ($P<0.01$) while no relationship between fish size and total fecundity was observed in homozygous red *O. niloticus*. The relationship between total fecundity and weight were much weaker than the relationship between total fecundity and length in homozygous wild type ($P<0.05$) and pooled data from all colour genotypes of *O. niloticus* ($P<0.05$).

2) Relative fecundity (Fig. 4.3.): Very significant negative relationships were found between relative fecundity and both fish weight and length in homozygous red ($P<0.001$), heterozygous red ($P<0.001$) and pooled data from all colour genotypes of *O. niloticus* ($P<0.001$) while homozygous wild type tilapia showed a weaker negative relationship between relative fecundity and both fish weight and length ($P<0.05$).

3) EW: BW ratio (Fig.4.5.): Significant negative relationships were detected between EW:BW ratio and fish weight in pooled data from all colour genotypes ($P<0.001$), homozygous red ($P<0.05$) and heterozygous ($P<0.01$) and between EW:BW and fish length in homozygous red ($P<0.05$) and pooled data from all colour genotypes of *O. niloticus* ($P<0.01$). No relationships were observed between EW:BW and fish weight in homozygous wild type and between EW:BW and fish length in homozygous wild type and heterozygous red *O. niloticus*.

Table 4.5. Linear regression (y=a+bx) analysis of reproductive traits of homozygous red (RR) *O. niloticus* (based on spawned fish). N is the number of data points, ns: not significant, *: P<0.05, **: P<0.01 and ***: P<0.001.

	UNTRANSFORMED DATA				Log10 TRANSFORMED DATA		
Dependent-Independent (y) (x)	N	Intercept (a)	Slope (b)	r	Intercept (a)	Slope (b)	r
Total fecundity-fish weight (g)	60	972	0.685	0.151 ns	2.64	0.172	0.172 ns
Total fecundity-fish length (cm)	60	432	35.3	0.226 ns	2.15	0.691	0.234 ns
Relative fecundity (egg/g)-fish weight (g)	60	9.27	-18.1×10^{-3}	-0.674 ***	2.66	-0.836	-0.655 ***
Relative fecundity (egg/g)-fish length (cm)	60	16.1	-0.560	-0.603 ***	3.53	-2.200	-0.580***
Egg weight (mg)-fish weight (g)	5	2.91	6.1×10^{-4}	0.137 ns	0.305	0.075	0.185 ns
Egg weight (g)- fish length (cm)	5	2.35	34.8×10^{-3}	0.228 ns	0.131	0.270	0.258 ns
Egg diameter (mm)-fish weight (g)	5	2.42	6.3×10^{-4}	0.168 ns	0.253	0.066	0.155 ns
Egg diameter (mm)- fish length (cm)	5	1.76	39.8×10^{-3}	0.311 ns	-0.043	0.345	0.316 ns
Egg volume (mm^3)-fish weight (g)	5	6.72	0.0043	0.131 ns	0.48	0.168	0.130 ns
Egg volume (mm^3)- fish length (cm)	5	1.5	0.304	0.270 ns	-0.39	0.960	0.290 ns
Total fecundity-egg volume (mm^3)	5	1517	-39.5	-0.380 ns	3.29	-0.245	-0.373 ns
EW:BW ratio-fish weight (g)	5	30.4	-59.7×10^{-3}	-0.908*	57.0	-1.410	-0.862 *
EW:BW ratio- fish length (cm)	5	56.1	-2.01	-0.895 *	6.05	-3.720	-0.880 *
Total egg volume (mm^3)-fish weight (g)	5	12553	-11	-0.274 ns	4.74	-0.318	-0.255 ns
Total egg volume (mm^3)- fish length (cm)	5	14269	-227	-0.166 ns	4.62	-0.490	-0.152 ns

Table 4.6. Linear regression (y=a+bx) analysis of reproductive traits of heterozygous red (Rr) *O. niloticus* (based on spawned fish). N is the number of data points, ns: not significant, *: P<0.05, **: P<0.01 and ***: P<0.001.

	UNTRANSFORMED DATA				Log10 TRANSFORMED DATA		
Dependent-Independent (y) (x)	N	Intercept (a)	Slope (b)	r	Intercept (a)	Slope (b)	r
Total fecundity-fish weight (g)	91	981	0.546	0.114 ns	2.51	0.219	0.157 ns
Total fecundity-fish length (cm)	91	422	33.7	0.193 ns	1.79	0.947	0.230 *
Relative fecundity (egg/g)-fish weight (g)	91	8.27	-14.6×10^{-3}	-0.580 ***	2.50	-0.777	-0.490***
Relative fecundity (egg/g)-fish length (cm)	91	13.5	-0.433	-0.472 ***	2.98	-1.78	-0.381***
Egg weight (mg)-fish weight (g)	5	5.30	-5.5×10^{-3}	-0.635 ns	1.65	-0.442	-0.601 ns
Egg weight (g)- fish length (cm)	5	8.76	-0.236	-0.772 ns	2.50	-1.45	-0.747 ns
Egg diameter (mm)-fish weight (g)	5	2.71	154×10^{-4}	0.130 ns	0.404	0.0150	0.122 ns
Egg diameter (mm)- fish length (cm)	5	2.33	19.5×10^{-3}	0.469 ns	0.244	0.147	0.453 ns
Egg volume (mm^3)-fish weight (g)	5	8.15	3.78×10^{-3}	0.372 ns	0.674	0.119	0.379 ns
Egg volume (mm^3)- fish length (cm)	5	4.32	0.228	0.641 ns	0.263	0.527	0.636 ns
Total fecundity-egg volume (mm^3)	5	-244	158	0.481 ns	1.96	1.16	0.472 ns
EW:BW ratio-fish weight (g)	5	40.4	-83.7×10^{-3}	-0.948 **	4.81	-1.47	-0.959 **
EW:BW ratio- fish length (cm)	5	71.0	-2.57	-0.832 ns	5.68	-3.38	-0.832 ns
Total egg volume (mm^3)-fish weight (g)	5	9708	5.7	0.146 ns	3.83	0.090	0.094 ns
Total egg volume (mm^3)- fish length (cm)	5	-2506	638	0.468 ns	2.61	1.08	0.425 ns

Table 4.7. Linear regression (y=a+bx) analysis of reproductive traits of homozygous wild type (rr) *O. niloticus* (based on spawned fish). N is the number of data points, ns: not significant, *: P<0.05, **: P<0.01 and ***: P<0.001.

	UNTRANSFORMED DATA				Log10 TRANSFORMED DATA		
Dependent-Independent (y) (x)	N	Intercept (a)	Slope (b)	r	Intercept (a)	Slope (b)	r
Total fecundity-fish weight (g)	68	543	1.78	0.350 **	1.69	0.539	0.336 *
Total fecundity-fish length (cm)	68	-449	-70.6	0.360**	0.668	1.76	0.371**
Relative fecundity (egg/g)-fish weight (g)	68	5.82	$-7x10^{-3}$	-0.376**	1.67	-0.453	-0.285*
Relative fecundity (egg/g)-fish length (cm)	68	9.04	-0.250	-0.331**	1.94	-1.04	-0.221*
Egg weight (mg)-fish weight (g)	5	4.31	$-3.07x10^{-3}$	-0.232 ns	1.25	-0.293	-0.26 ns
Egg weight (g)- fish length (cm)	5	7.46	-0.189	-0.290 ns	2.16	-1.22	-0.289 ns
Egg diameter (mm)-fish weight (g)	5	2.68	$-2.5x10^{-4}$	-0.064 ns	0.514	-0.040	-0.079 ns
Egg diameter (mm)- fish length (cm)	5	2.94	-0.016	-0.083 ns	0.61	-0.142	-0.087 ns
Egg volume (mm^3)-fish weight (g)	5	8.21	$-5x10^{-3}$	-0.015 ns	1.00	$-4.2x10^{-3}$	-0.028 ns
Egg volume (mm^3)- fish length (cm)	5	9.1	-0.048	-0.028 ns	1.10	-0.15	-0.031 ns
Total fecundity-egg volume (mm^3)	5	1955	-103	-0.675 ns	3.67	-0.693	-0.690 ns
EW:BW ratio-fish weight (g)	5	16.1	$-14.1x10^{-3}$	-0.424 ns	2.05	-0.395	-0.413 ns
EW:BW ratio- fish length (cm)	5	27.6	-0.730	-0.448 ns	2.99	-1.44	-0.462 ns
Total egg volume (mm^3)-fish weight (g)	5	1661	22.7	0.746 ns	3.60	$1.1x10^{-3}$	0.737 ns
Total egg volume (mm^3)- fish length (cm)	5	-16142	1145	0.766 ns	0.10	2.87	0.758 ns

Table 4.8. Linear regression (y=a+bx) analysis of pooled reproductive traits data of homozygous red, heterozygous red and homozygous wild type *O. niloticus* (based on spawned fish). N is the number of data points, ns: not significant, *: P<0.05, **: P<0.01 and ***: P<0.001.

	UNTRANSFORMED DATA				Log10 TRANSFORMED DATA		
Dependent-Independent (y) (x)	N	Intercept (a)	Slope (b)	r	Intercept (a)	Slope (b)	r
Total fecundity-fish weight (g)	219	956	0.557	0.123 ns	2.60	0.179	0.142 *
Total fecundity-fish length (cm)	219	476	30.20	0.183 **	2.04	0.755	0.203 **
Relative fecundity (egg/g)-fish weight (g)	219	8.35	-0.0152	-0.618 **	2.61	-0.824	-0.549 ***
Relative fecundity (egg/g)-fish length (cm)	219	14.4	-0.483	-0.540 **	3.32	-2.05	-0.461 ***
Egg weight (mg)-fish weight (g)	15	3.56	-0.00075	-0.079 ns	0.620	-0.040	-0.049 ns
Egg weight (g)- fish length (cm)	15	4.06	-0.034	-0.093 ns	0.741	-0.166	-0.072 ns
Egg diameter (mm)-fish weight (g)	15	2.55	$3.46x10^{-4}$	0.100 ns	0.323	0.040	0.101 ns
Egg diameter (mm)- fish length (cm)	15	1.95	0.0325	0.246 ns	0.057	0.274	0.247 ns
Egg volume (mm^3)-fish weight (g)	15	7.26	$3.86x10^{-3}$	0.128 ns	0.532	0.157	0.135 ns
Egg volume (mm^3)- fish length (cm)	15	102	0.298	0.260 ns	-0.23	0.865	0.266 ns
Total fecundity-egg volume (mm^3)	15	1464	-32.8	-0.261 ns	3.27	-0.219	-0.270 ns
EW:BW ratio-fish weight (g)	15	29.3	$-52.7x10^{-3}$	-0.762 ***	3.95	-1.14	-0.761 ***
EW:BW ratio- fish length (cm)	15	54.1	-1.88	-0.715 **	5.17	-3.04	-0.724 **
Total egg volume (mm^3)-fish weight (g)	15	9232	2.40	0.055 ns	3.86	0.054	0.042 ns
Total egg volume (mm^3)- fish length (cm)	15	2602	340	0.207 ns	3.09	0.676	0.188 ns

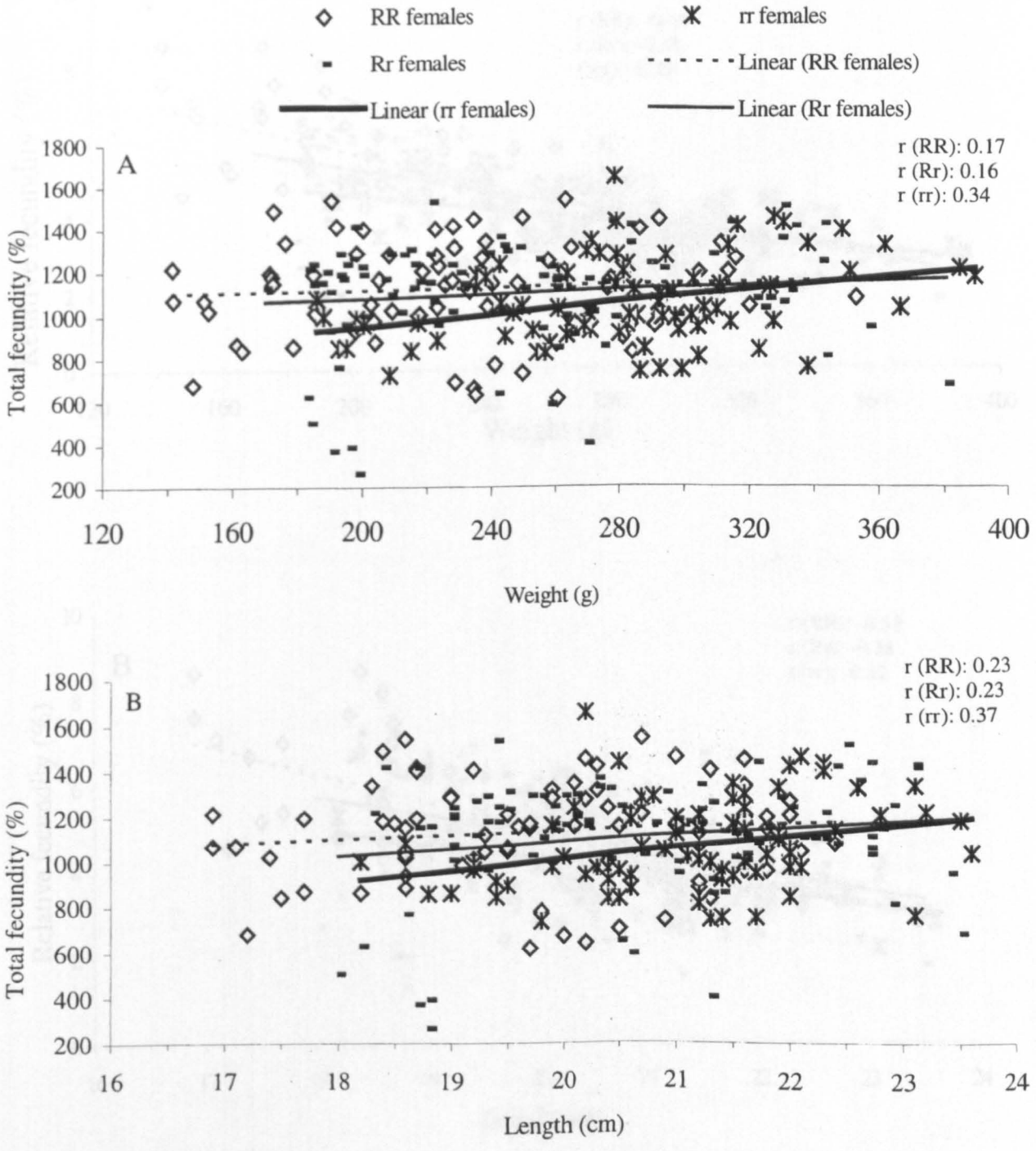


Figure 4.2. Relationships between (A) total fecundity and fish weight (g), (B) total fecundity and fish length (cm).in three colour genotypes (RR, rr, and Rr) of *O. niloticus* (based on spawned fish).

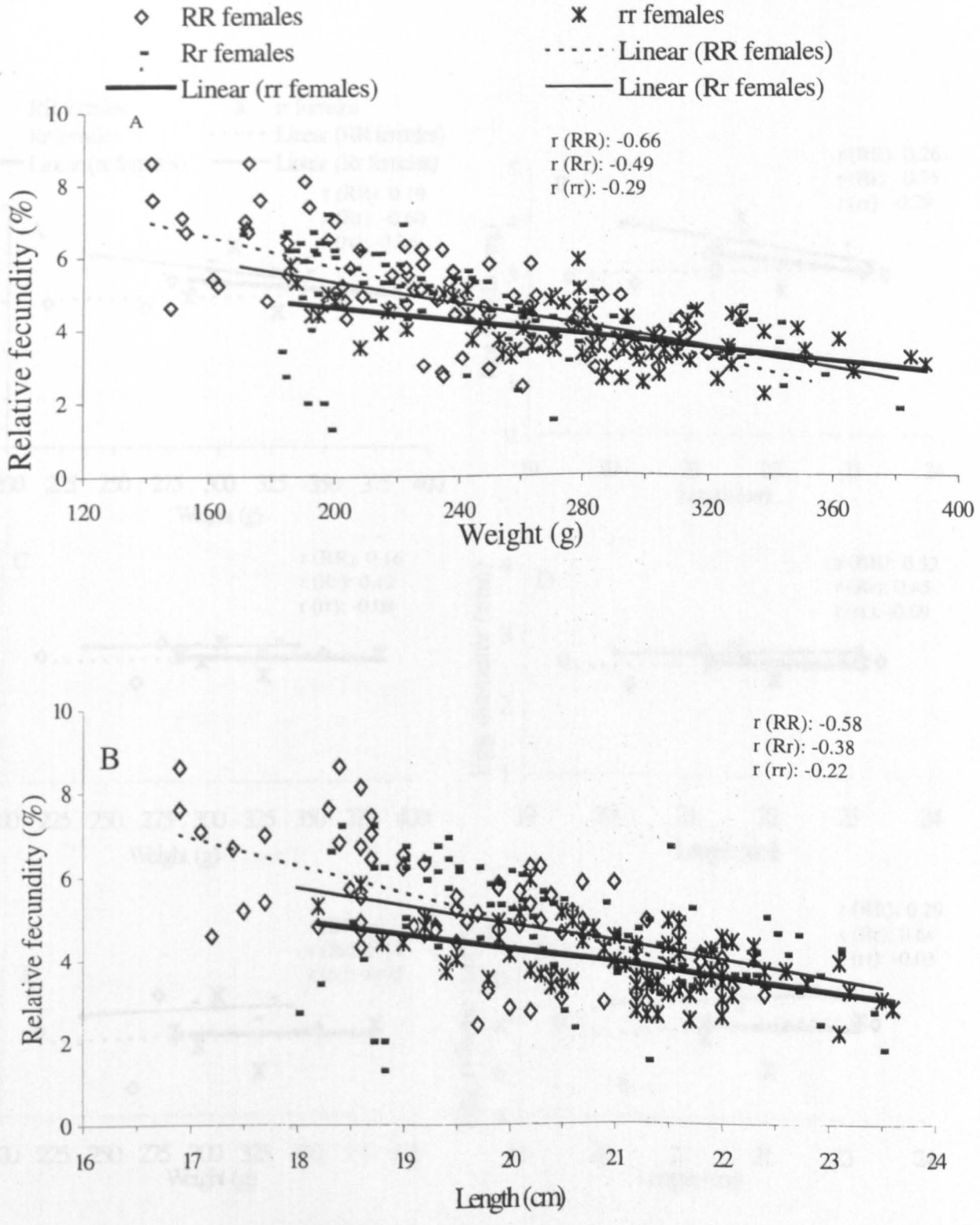


Figure 4.3. Relationships between (A) relative fecundity and fish weight (g), (B) relative fecundity and fish length (cm).in three colour genotypes (RR, rr, and Rr) of *O. niloticus* (based on spawned fish).

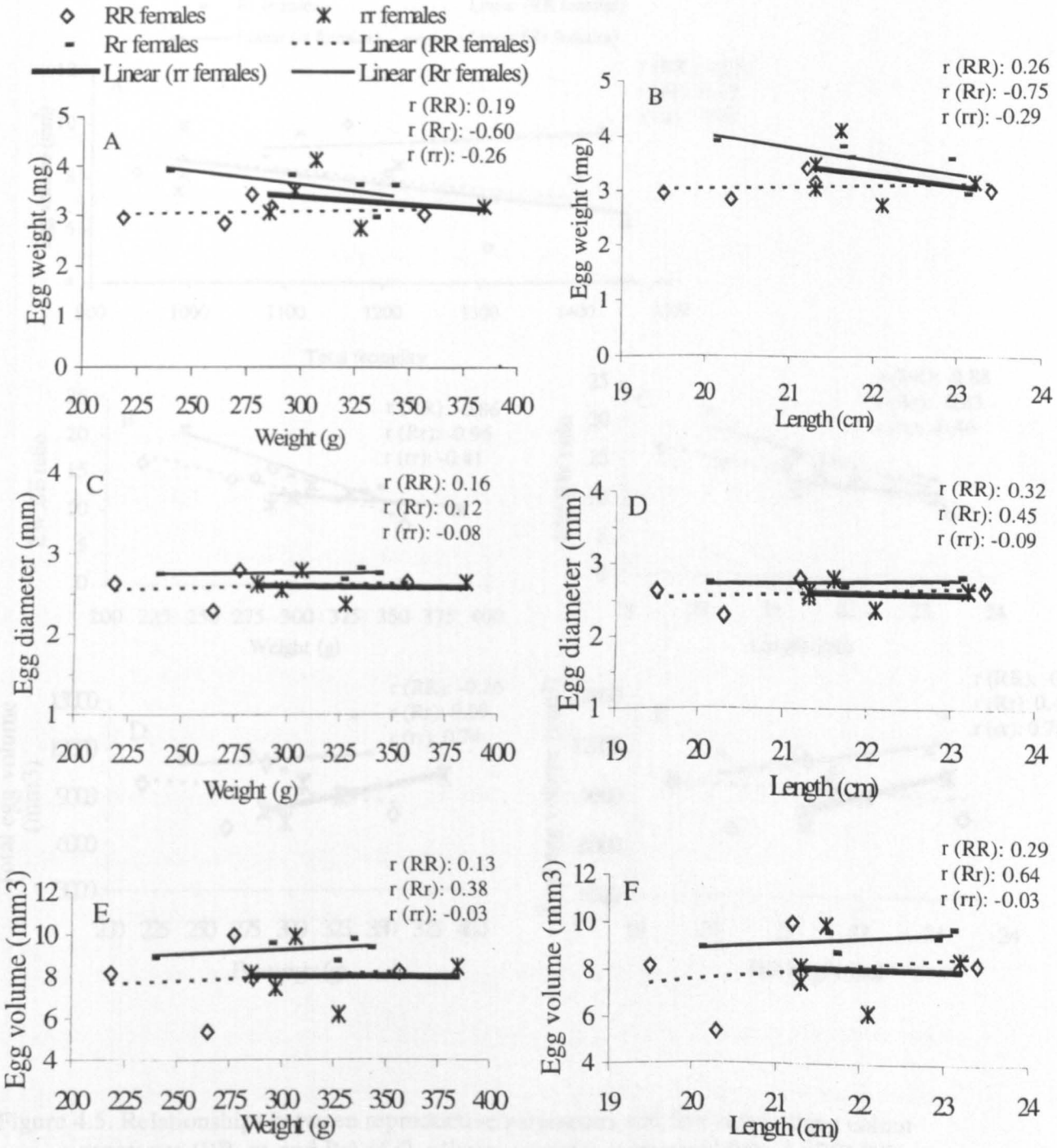


Fig. 4.4. Relationships between reproductive parameters and fish size in three colour genotypes (RR, rr, and Rr) of *O. niloticus* (based on spawned fish).A) egg weight and fish weight, B) egg weight and fish length, C) Egg diameter and fish weight, D) Egg diameter and fish length, E) Egg volume and fish weight, F) Egg diameter and fish length.

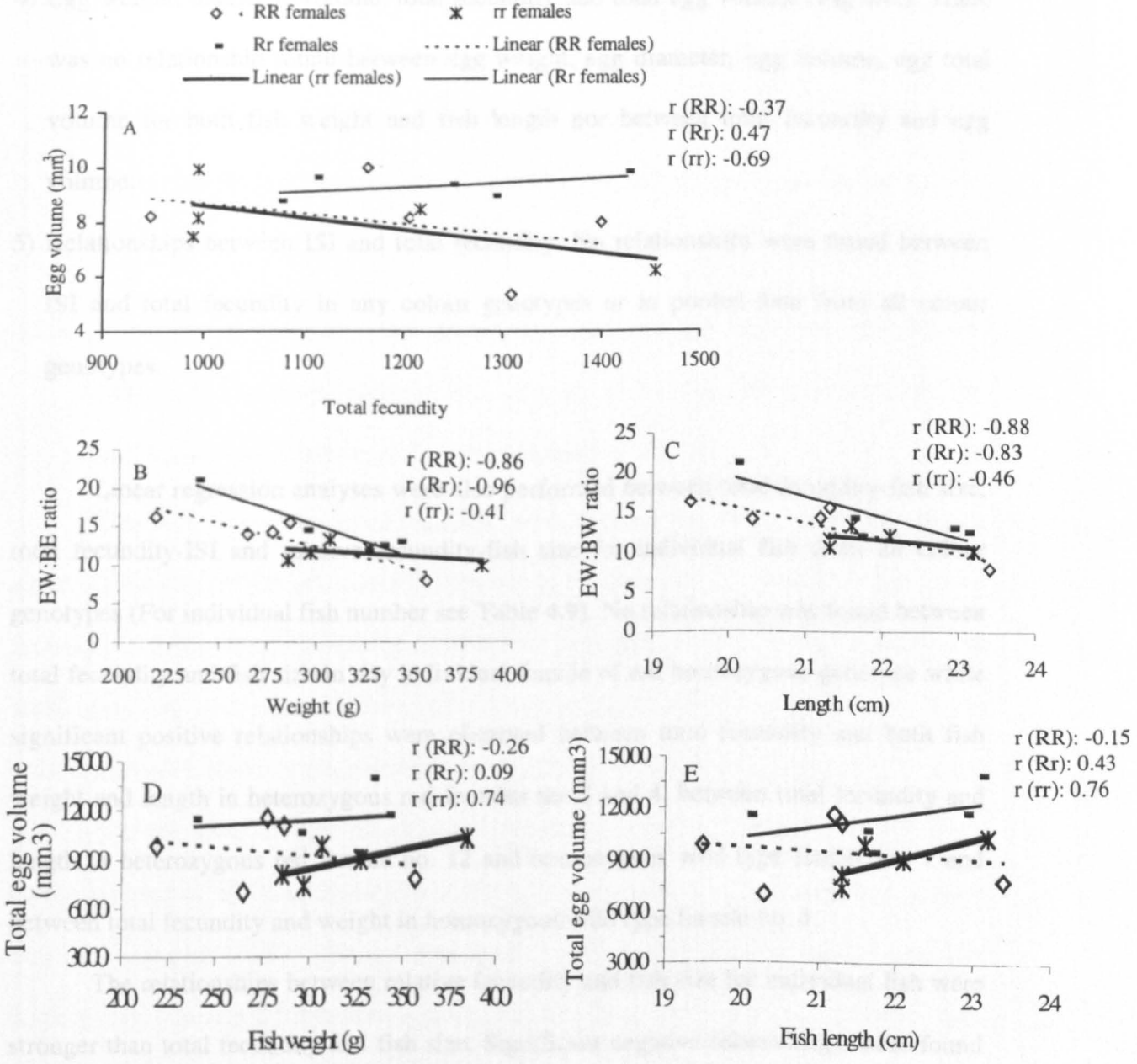


Figure 4.5. Relationships between reproductive parameters and fish size in three colour genotypes (RR, rr, and Rr) of *O. niloticus* (based on spawned fish. A) EW:BW ratio and total fecundity, B) EW:BW ratio and fish weight, C) EW:BW ratio and fish length, D) Total egg volume and fish weight, E) Total egg volume and fish length.

4) Egg weight, diameter, volume, total fecundity and total egg volume (Fig 4.4.): There was no relationship found between egg weight, egg diameter, egg volume, egg total volume for both fish weight and fish length nor between total fecundity and egg volume.

5) Relationships between ISI and total fecundity: No relationships were found between ISI and total fecundity in any colour genotypes or in pooled data from all colour genotypes.

Linear regression analyses were also performed between total fecundity-fish size, total fecundity-ISI and relative fecundity-fish size for individual fish from all colour genotypes (For individual fish number see Table 4.9). No relationship was found between total fecundity and fish size in any individual female of red homozygous genotype while significant positive relationships were observed between total fecundity and both fish weight and length in heterozygous red females no. 3 and 4, between total fecundity and length in heterozygous red female no. 12 and homozygous wild type female no. 7 and between total fecundity and weight in homozygous wild type female no. 4.

The relationships between relative fecundity and fish size for individual fish were stronger than total fecundity and fish size. Significant negative relationships were found between relative fecundity and both fish weight and length for homozygous red female no.2, homozygous wild type females no's 1, 2, 3 and 5 and for heterozygous red females of no's 3, 4, 6, 9, 10, 12 and 13. Homozygous red females no's 3 and 9, homozygous wild type females no's 6, 8 and 10 and heterozygous red female of no. 1 showed significant negative relationship between relative fecundity and fish weight while

homozygous red female of no. 4 and heterozygous red females no's 1 and 2 showed significant negative relationship between relative fecundity and fish length.

Regression analysis between ISI and total fecundity showed significant negative relationships only for homozygous red female no. 9 and heterozygous red female no. 10.

4.3.5. Variation of fecundity and inter-spawning-interval (ISI) over consecutive spawns in serial spawning of homozygous red (RR), heterozygous red (Rr) and homozygous wild type (rr) *O. niloticus*

This investigation analyzed data from several serial-spawning females of different colour genotypes of *O. niloticus*. The variation in terms of ISI, weight, length, total fecundity and relative fecundity of three colour genotypes over consecutive spawns in serial spawning is depicted in Table 4.9. Definite trends over successive spawns were only observed in terms of body weight and length of all colour genotypes. Body weight and length were found to increase steadily with each successive spawn in each fish of all colour genotypes. ISI exhibited quite marked variation with successive spawns in each fish (CV ranged 14.3-77.3% for homozygous red in RR female no's 8 and 10, respectively, 7.5-61.9% for heterozygous red in Rr female no's 1 and 6, respectively and 10.5-78.1% for homozygous wild type in rr female no's 3 and 8, respectively).

Relative fecundity was also found to exhibit variation with successive spawns but to a lesser extent than ISI (CV ranged 11.5-29.2% for homozygous red in RR female no. 8 and 1, respectively, 7.5-26.6% for heterozygous red in Rr female no's 3 and 13, respectively and 1.1-52.5% for homozygous wild type in female no's 9 and 1, respectively.).

The variation in terms of total fecundity exhibited lower variation in homozygous wild type tilapia (CV range 0.3-14.2% in rr female no. 9 and 7, respectively) than both homozygous red (CV 9.8-28.1% in RR female no. 6 and 1, respectively) and heterozygous red tilapia (CV 4.1-30.9% in Rr female no's 2 and 5, respectively).

4.3.6. Survival rates in crosses involving different colour genotypes of *O. niloticus*

Mean survival rates in crosses of homozygous red females and homozygous red males (RR x RR), homozygous red and homozygous wild type (RR x rr), homozygous wild type and homozygous red (rr x RR) *O. niloticus* at pigmentation, hatching and yolk sac stages are shown in Table 4.10. and are graphically presented in Fig. 4.6.

There were no significant differences between colour genotypes at any developmental stages. General linear model (GLM) found no female, male or female*male interaction effects on the survival rates of any developmental stages within colour genotypes. Significant differences were found between stages when all the data from all crosses were pooled. This showed the best pigmentation > hatching > yolk sac indicating that mortality increased with time.

Table 4.9. Variation of fish weight, length, fecundity, relative fecundity and inter spawning interval (ISI) over successive spawns in homozygous red (RR), heterozygous red (Rr) and homozygous wild type (rr) *O. niloticus*. C.V: Coefficient of variance (see Section 4.2.4.).

	FISH 1 Homozygous red (RR)					FISH 2 Homozygous red (RR)					FISH 3 Homozygous red (RR)				
Spawn no	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)
1	?	234.0	19.6	1150	4.92	?	?	?	?	?	?	178.6	18.2	858	4.80
2	12	234.4	20.0	667	2.85	24	?	?	?	?	18	176.2	18.3	1337	7.59
3	19	235.0	20.2	636	2.71	?	141.4	16.9	1215	8.59	20	172.6	18.4	1487	8.62
4	32	227.9	20.3	1415	6.21	13	141.7	16.9	1071	7.56	24	172.0	18.4	1177	6.84
5	?	223.4	20.4	1230	5.51	15	152.6	17.4	1022	6.70	?	172.4	18.6	1148	6.65
6	16	228.6	20.5	695	3.04	15	171.4	17.7	1193	6.96	19	184.8	18.7	1190	6.44
7	16	236.5	20.6	1203	5.09	12	190.4	18.6	1537	8.07	18	198.0	19.0	1288	6.51
8	18	249.5	21.0	1453	5.82	12	191.9	18.7	1412	7.36	20	208.1	19.0	1282	6.16
9	15	248.4	21.0	?	?	21	200.0	18.7	1400	7.00	27	218.4	19.5	1205	5.52
10	46	277.0	21.0	?	?	36	222.4	19.2	1402	6.30	29	227.7	19.7	1163	5.11
11	18	?	?	?	?	24	233.0	19.3	1115	4.79	21	228.3	19.9	1313	5.75
12	30	276.3	21.2	1120	4.05	22	238.6	19.5	1044	4.38	21	222.8	19.9	1275	5.72
13	25	?	?	?	?	23	264.8	20.3	1308	4.94	22	236.9	20.2	1266	5.34
14	22	313.2	22.0	1200	3.83	25	?	?	?	?	24	?	?	?	?
C.V (%)	42.7	10.8	3.1	28.1	29.2	35.2	21.2	6.0	14.0	20.9	15.9	12.4	3.7	11.7	16.9
Average ISI ±SE			22.4±2.8			20.17±2.1					21.92±1.0				

	FISH 4 Homozygous red (RR)					FISH 5 Homozygous red (RR)					FISH 6 Homozygous red (RR)				
Spawn no	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)
1	?	205.0	18.5	1168	5.70	?	?	?	?	?	?	?	?	?	?
2	20	249.6	20.9	735	2.95	19	?	?	?	?	17	?	?	?	?
3	21	281.0	21.2	898	3.20	11	?	?	?	?	25	240.0	20.1	1150	4.79
4	21	284.0	21.3	830	2.92	?	203.4	19.1	982	4.83	17	238.0	20.1	1340	5.63
5	?	278.9	21.8	1140	4.09	11	?	?	?	?	?	239.6	20.3	?	?
6	30	283.3	21.8	954	3.37	31	?	?	?	?	23	255.4	21.0	?	?
7	23	304.3	21.8	1193	3.92	26	?	?	?	?	36	177.2	21.2	1163	4.20
8	33	319.9	22.1	1042	3.26	27	?	?	?	?	58	292.0	21.6	1443	4.94
9	96	352.9	22.4	1077	3.05	27	?	?	?	?	43	311.0	21.6	1332	4.28
10	54	387.7	23.4	?	?	22	?	?	?	?					
C.V(%)	70.5	17.4	5.9	15.9	24.5	34.7					48.6	17.2	3.2	9.8	12.1
Average ISI ±SE			37.3±9.3			21.8±2.7					31.3±5.7				

Table 4.9. continued

	FISH 7 Homozygous red (RR)					FISH 8 Homozygous red (RR)					FISH 9 Homozygous red (RR)				
Spawn no	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)
1	?	?	?	?	?	?	?	?	?	?	?	162.9	17.5	840	5.16
2	12	?	?	?	?	15	?	?	?	?	23	179.0	17.7	?	?
3	13	?	?	?	?	?	147.8	17.2	680	4.60	25	197.8	18.6	935	4.73
4	?	151.2	17.1	1070	7.07	27	161.3	17.7	866	5.37	29	209.0	18.6	1027	4.91
5	7	202.5	19.5	1057	5.22	24	185.4	18.6	1014	5.47	24	217.6	19.2	1008	4.63
6	24	?	?	?	?	33	203.8	18.6	881	4.32	26	223.1	19.3	1041	4.67
7	14	209.9	19.0	775	3.69						53	260.6	19.7	614	2.36
8	11	249.6	20.4	?	?						34	292.2	20.4	995	3.41
9	7	248.0	20.5	1147	4.63										
10	19	?	?	?	?										
11	26	260.0	20.7	?	?										
12	22	267.2	21.2	?	?										
13	28	?	?	?	?										
14	26	286.2	21.3	1398	4.88										
15	29	315.6	22.0	1263	4.00										
16	15	307.4	22.1	?	?										
C.V(%)	42.8	20.2	7.4	18.9	24.3	30.3	14.3	3.9	16.0	11.5	34.6	19.3	5.2	23.6	
Average ISI ±SE			18.1±2.1			24.8±4.3					30.6±4.0				

	FISH 10 Homozygous red (RR)					FISH 1 Heterozygous red (Rr)					FISH 2 Heterozygous red (Rr)				
Spawn no	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)
1	?	?	?	?		?	260.0	21.3	851	3.27	?	?	?	?	?
2	12	225.5	19.7	1145	5.08	?	265.0	21.3	910	3.43	37	196.8	18.7	1412	7.17
3	?	234.3	20.2	1445	6.17	45	303.6	22.6	?	?	30	?	?	?	?
4	32	263.0	20.7	1536	5.84	40	343.2	22.9	810	2.36	?	?	?	?	?
5	22	257.6	20.7	1250	4.85	46	356.6	23.4	946	2.65	25	214	19.5	1306	6.10
6	28	262.7	20.7	1200	4.57	40	380.4	23.5	683	1.80	22	231.2	20.3	?	?
7	82	290.8	21.6	951	3.27						33	?	?	?	?
8											56	282.5	21.2	1330	4.71
C.V(%)	77.3	9.1	3.1	16.8	20.7	7.5	15.7	4.4	12.2	24.7	35.8	16.0	5.38	4.1	20.6
Average ISI ±SE			35.2±12.2			42.8±1.6					33.8±5.0				

Table 4.9. continued

	FISH 3 Heterozygous red (Rr)					FISH 4 Heterozygous red (Rr)					FISH 5 Heterozygous red (Rr)				
Spawn no	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)
1	?	182.8	18.3	1215	6.65	?	185.4	18.6	?	?	?	183.7	18.0	505	2.75
2	22	186.6	18.8	1152	6.17	23	184.2	18.7	1148	6.23	14	190.6	18.7	373	1.96
3	23	92.5	19.0	1191	6.19	24	188.2	19.0	1204	6.40	34	?	?	?	?
4	17	196.4	19.2	1265	6.44	?	189.3	19.2	1114	5.88	16	198.4	18.8	265	1.35
5	?	192.3	19.3	1287	6.69	21	199.0	19.5	1227	6.17	?	195.9	18.8	392	2.00
6	17	206.2	19.4	1171	5.67	19	207.8	19.7	1287	6.19	18	?	?	?	?
7	18	216.6	19.9	?	?	21	212.0	20.0	1284	6.06	19	?	?	?	?
8	21	237.3	20.7	?	?	23	220.0	20.0	1154	5.25	25	241.5	20.5	642	2.66
9	42	269.7	21.3	1402	5.20	64	?	?	?	?	19	258.1	20.6	587	2.27
10	54	?	?	?	?	18	229.4	20.1	1198	5.22	23	267.9	20.7	?	?
11	28	322.3	21.6	?	?	20	238.4	20.1	1289	5.41	21	272.7	21.1	?	?
12	36	332.8	22.8	?	?	20	241.9	20.3	1362	5.63	38	252.0	21.3	?	?
13											17	?	?	?	?
14											23	?	?	?	?
15											21	298.1	21.7	?	?
C.V(%)	44.7	30.7	7.0	7.0	8.8	54.3	10.3	3.1	6.4	7.5	31.2	17.2	6.6	30.9	23.8
Average ISI ±SE			27.8±3.9			25.3±4.3					22.2±1.9				

	FISH 6 Heterozygous red (Rr)					FISH 7 Heterozygous red (Rr)					FISH 8 Heterozygous red (Rr)				
Spawn no	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)
1	?	221.0	19.4	1528	6.91	?	197.3	18.4	1415	7.17	?	182.5	18.2	627	3.43
2	22	220.0	19.8	1286	5.85	20	199.6	19	1004	5.03	36	188.9	18.5	933	4.94
3	24	215.0	19.9	1131	5.26	22	?	?	?	?	15	191.8	18.6	765	3.99
4	14	217.0	19.9	1160	5.35	15	199.3	20.1	1190	5.97	?	194.3	19.4	1238	6.37
5	?	215.3	19.9	1238	5.75	?	211.3	20.7	1020	4.83	19	208.2	19.8	1100	5.28
6	18	230.5	20.2	1146	4.97	28	207.0	20.8	?	?	22	226.8	20.5	1235	5.44
7	19	243.1	20.3	1322	5.44	21	243.9	20.9	1296	5.31	25	249.0	20.6	?	?
8	25	248.0	20.4	1313	5.29	21	245.3	21.2	?	?	50	274.6	21.4	855	3.15
9	22	259.3	20.8	1158	4.47	20	258.3	21.2	1222	4.73	26	281.6	21.5	?	?
10	73	270.2	21.6	1026	3.80	23	261.6	21.2	?	?	25	?	?	?	?
11	26	294.4	21.6	1110	3.77	19	263.8	21.5	1117	4.23	25	?	?	?	?
12	26	307.8	21.9	1218	4.15	43	?	?	?	?	29	330.1	22.4	1507	4.56
13						44	286.5	21.7	?	?					
14						22	300.4	22	?	?					
C.V(%)	61.9	13.0	4.0	10.8	18.1	37.1	15.0	5.2	12.5	18.3	35.9	21.3	7.1	28.1	23.4
Average ISI ±SE			26.9±5.3			24.8±2.7					27.2±3.1				

Table 4.9. continued

	FISH 9 Heterozygous red (Rr)					FISH 10 Heterozygous red (Rr)					FISH 11 Heterozygous red (Rr)				
Spawn no	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)
1	?	221.1	19.0	1070	4.84	?	255.2	21.0	1178	4.62	?	242.3	20.3	1020	4.21
2	45	222.5	19.3	961	4.32	25	249.5	21.0	1127	4.52	?	?	?	?	?
3	14	?	?	?	?	30	255.0	21.0	940	3.69	20	254.2	21.3	1254	4.93
4	?	227	19.7	1022	4.50	?	?	?	?	?	21	259.7	21.4	1127	4.33
5	23	259.5	20.2	1170	4.51	28	?	?	?	?	27	277.1	21.7	1432	5.17
6	47	265.2	20.4	1000	3.77	28	306.0	22.3	1097	3.58	31	289.0	22.0	1234	4.27
7	25	277.9	21.0	987	3.55	28	324.0	22.7	1030	3.18	44	306.5	22.1	1102	3.59
8	29	?	?	?	?	26	329.6	22.7	1057	3.21	16	321.6	22.4	1075	3.34
9	28	?	?	?	?	24	332.3	22.7	1107	3.33	31	282.5	22.4	1414	5.00
10	38	325.6	21.7	1075	3.30	23	326.3	22.8	1140	3.49	39	?	?	?	?
11						25	341.9	22.9	1245	3.58					
12						26	347.5	23.0	?	?					
C.V(%)	36.5	14.7	4.7	6.8	14.0	8.2	12.6	3.9	8.0	14.3	33.7	9.6	3.2	12.8	15.2
Average ISI ±SE			31.1±4.0			26.3±0.7					28.6±3.4				

	FISH 12 Heterozygous red (Rr)					FISH 13 Heterozygous red (Rr)					FISH 1 Homozygous wild type (rr)				
Spawn no	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)
1	?	269.5	21.2	1161	4.31	?	183.9	19.0	1237	6.73	?	187.9	18.2	1000	5.32
2	29	271.0	21.5	1012	3.73	42	194.0	19.2	965	4.97	30	192.6	18.8	852	4.42
3	24	169.9	21.5	1130	6.65	16	193.3	19.3	1173	6.07	15	195.0	19.0	857	439
4	?	274.7	21.8	1147	4.18	?	?	?	?	?	20	198.0	19.2	989	4.99
5	21	292.7	22.3	1210	4.13	25	?	?	?	?	?	208.3	19.8	730	3.50
6	27	313.4	22.7	1432	4.57	34	261.3	21.1	1165	4.46	26	?	?	?	?
7	26	333.3	23.1	1411	4.23	68	297.3	21.6	1000	3.36	25	217.1	19.9	971	4.47
8	34	342.5	23.1	1423	4.15	29	309.8	22.2	1101	3.55	18	258.1	20.6	870	3.37
9	43	?	?	?	?	28	329.1	22.6	1354	4.11	34	270.0	21.1	1013	3.75
10	53	?	?	?	?						33	285.4	21.3	994	3.48
11											33	299.2	21.7	748	2.51
C.V(%)	33.7	19.0	3.4	12.9	20.1	48.5	24.4	7.4	11.8	26.6	27.2	18.4	5.9	11.8	52.5
Average ISI ±SE			32.1±3.8			34.6±6.3					26.0±2.4				

Table 4.9. continued

	FISH 2 Homozygous wild type (rr)					FISH 3 Homozygous wild type (rr)					FISH 4 Homozygous wild type (rr)				
Spawn no	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)
1	?	185.9	18.6	1079	5.80	?	?	?	?	?	?	264.0	20.3	962	3.64
2	39	200.1	19.2	961	4.80	13	278.4	20.2	1647	5.91	25	268.1	20.4	960	3.58
3	45	215.0	19.4	840	3.91	23	278.9	20.5	1430	5.13	25	263.5	20.4	910	3.45
4	?	223.1	19.5	890	3.99	19	279.0	20.7	1274	4.57	?	270.8	20.6	929	3.43
5	15	234.4	19.9	1155	4.93	20	273.7	20.8	1280	4.68	21	287.7	21.2	845	2.94
6	25	249.5	20.9	1043	4.18	?	284.5	21.0	1115	4.00	20	302.6	21.5	1161	3.84
7	6	263.6	21.0	1187	4.50	21	294.1	21.5	1272	4.33	18	298.1	21.5	930	3.12
8	15	260.7	21.2	1030	3.95	20	313.2	21.9	1321	4.22	42	306.6	21.6	994	3.24
9	21	270.0	21.3	?	?	25	316.1	22.0	1416	4.48	20	309.8	21.8	1026	3.31
10	16	286.4	21.3	740	2.58	51	327.5	22.1	1455	4.44	17	309.1	21.9	?	?
11	20	292.4	21.4	747	2.55	21	330.3	22.3	1429	4.33	20	296.4	21.9	1108	3.74
12	24	304.0	21.7	945	3.11	28	?	?	?	?	18	313.8	21.9	?	?
13	29	327.4	22.0	971	2.97	22	348.5	22.3	1396	4.00	22	326.3	21.9	1130	3.46
14	20	334.4	22.1	?	?	21	361.8	22.6	1322	3.65					
15						20	361.6	22.8	?	?					
C.V(%)	46.9	17.5	5.5	14.9	25.4	38.4	10.5	4.0	9.6	13.1	30.9	7.1	3.0	10.1	7.8
Average ISI ±SE			22.9±3.1			23.4±2.5					22.6±2.1				

	FISH 5 Homozygous wild type (rr)					FISH 6 Homozygous wild type (rr)					FISH 7 Homozygous wild type (rr)				
Spawn no	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)
1	?	242.4	20.1	1234	5.09	?	269.0	20.5	951	3.53	?	280.0	21.4	915	3.27
2	17	235.1	20.6	1187	5.05	17	?	?	?	?	17	282.3	21.4	955	3.38
3	10	238.0	20.6	1178	4.95	22	281.0	21.2	1198	4.26	?	338	22.4	?	?
4	?	242.8	20.7	1057	4.35	16	283.0	21.2	1000	3.53	20	331.5	22.4	?	?
5	38	271.0	21.5	1370	5.06	?	292.0	22.0	1053	3.61	55	351	22.8	1200	3.42
6	22	269.6	21.6	1294	4.80	26	305.9	22.0	1033	3.38	15	354.0	23.0	?	?
7	24	280.8	21.6	1220	4.34	22	320.4	22.4	?	?	18	346.0	23.0	?	?
8	25	?	?	?	?	23	322.2	22.5	?	?	11	337.9	23.1	1330	3.94
9	20	282.3	21.6	1231	4.36	22	28.8	22.6	?	?	26	384.5	23.2	1215	3.16
10	22	278.7	21.6	1138	4.08	43	351.5	22.6	?	?	25	389.3	23.5	1177	3.02
11	24	292.1	21.9	1093	3.74	32	339.0	23.0	?	?					
12	23	310.7	22.4	1137	3.66	26	372.2	23.5	?	?					
13						27	377.2	23.9	?	?					
C.V(%)	31.2	9.3	3.2	7.5	11.7	29.7	30.9	4.4	8.9	9.4	58.6	10.7	3.2	14.3	9.4
Average ISI ±SE			22.5±2.2			25.1±2.2					23.4±4.8				

Table 4.9. continued

	FISH 8 Homozygous wild type (rr)					FISH 9 Homozygous wild type (rr)					FISH 10 Homozygous wild type (rr)				
Spawn no	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)	ISI	Weight (g)	Length (cm)	Total fecundity	Relative Fecundity (eggs/g)
1	?	244.2	19.4	908	3.72	?	254.1	20.4	830	3.27	?	322.9	22.0	840	2.60
2	15	246.7	20.0	1014	4.11	18	256.7	20.5	827	3.22	25	314.0	22.1	970	3.09
3	17	?	?	?	?	45	?	?	?	?	?	338.0	23.1	760	2.25
4	18	?	?	?	?	?	?	?	?	?	47	366.6	23.6	1033	2.82
5	17	252.8	20.2	932	3.69	65	321.3	21.7	?	?					
6	?	?	?	?	?										
7	89	302.0	21.2	1000	3.31										
8	33	304.3	21.2	808	2.66										
9	20	297.0	21.3	988	3.33										
10	22	293.3	21.3	990	3.38										
11	44	?	?	?	?										
C.V(%)	78.1	10.0	3.8	7.7	13.1	55.3	13.7	3.5	0.3	1.1	43.2	6.9	3.4	13.7	13.2
Average ISI ±SE			30.6±7.8			30.6±8.0					36.0±11.0				

Table 4.10. Mean survival rates in crosses of homozygous red (RR) x homozygous red (RR), homozygous red (RR) x homozygous wild type (rr), homozygous wild type (rr) x homozygous wild type (rr) and homozygous wild type (rr) x homozygous red (RR) *O. niloticus* at pigmentation, hatching and yolk sac stages. Same superscripts in the same column are not significantly different (P<0.05).

PARENTS			MEAN SURVIVAL RATE (%±SE)		
Female genotype	Male genotype	No. of crosses	Pigmentation stage	Hatching stage	Yolk sac stage
RR	RR	9	51.79±7.19[a]	32.63±16.11[a]	23.94±4.49[a]
RR	rr	6	62.17±6.47[a]	33.07±8.37[a]	24.68±7.08[a]
rr	rr	8	42.16±9.06[a]	34.99±8.03[a]	32.46±7.58[a]
rr	RR	6	44.06±11.12[a]	37.46±10.15[a]	32.84±8.66[a]
OVERALL			**49.80±4.28**	**34.37±3.67**	**28.28±3.32**

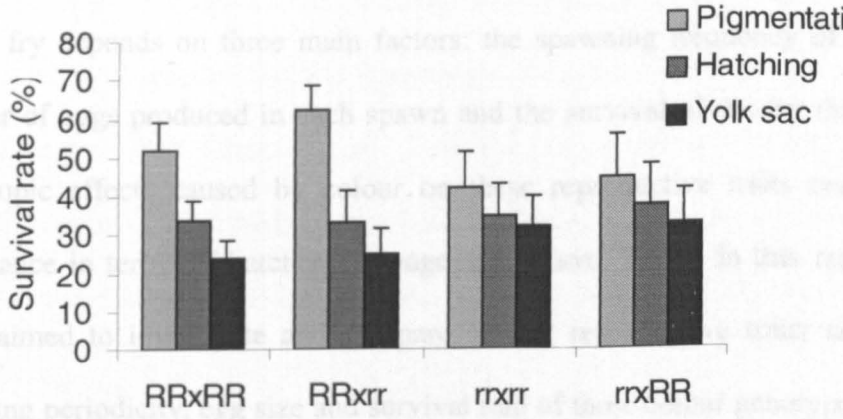


Figure 4.6. Mean survival rates in crosses of homozygous red (RR) x homozygous red (RR), homozygous red (RR) x homozygous wild type (rr), homozygous wild type (rr) x homozygous wild type (rr) and homozygous wild type (rr) x homozygous red (RR) *O. niloticus* at pigmentation, hatching and yolk sac stages.

4.4. Discussion

According to Bromage and Cumaranatunga (1988) the primary objective of hatcheries is to produce the maximum number of the highest quality eggs from the broodstock available. In this respect, intensive tilapia hatcheries are constrained through variation in fecundity and inter-spawning interval between individuals. The asynchronous nature of spawning in tilapia leads to a need for extensive facilities and time consuming management of large numbers of fish in order to supply the market demand for fry.

Increasing interest for culturing red morphs of tilapia stimulated research into of fingerling production. As in most cultured fish, nutrition (Chang *et al.*, 1988), crossbreeding (El-Gamal *et al.*, 1988), salinity (Watanabe *et al.*, 1989), season (Galman *et al.*, 1988), strain (Eguia, 1996; Romana-Eguia and Eguia, 1999) sex ratio and broodstock age affect reproduction in red tilapias (Smith *et al.*, 1991). The production of tilapia fry depends on three main factors: the spawning frequency of each female, the number of eggs produced in each spawn and the survival of the fry (Mires, 1980). Any pleiotropic effects caused by colour on these reproductive traits could have a great importance in terms of hatchery management (Tave, 1993). In this respect, the present study aimed to investigate and compare several reproductive traits such as fecundity, spawning periodicity, egg size and survival rate of three colour genotypes of *O. niloticus*. All the broodstock were held in the same environmentally controlled recirculating systems, minimizing variation in food availability, photoperiod and water temperature and quality. Although, almost all existing publications on reproductive traits of tilapia species originate from natural spawning of broodstocks in isolated ponds and lakes, the present study successfully used strip-spawning methods under laboratory conditions.

The present study showed no significant differences between colour genotypes of *O. niloticus* in terms of total fecundity, ISI, egg size and survival rate indicating no positive or negative pleiotropic effects of any colour morphs on these reproductive traits under these conditions. Therefore this discussion will be generally based on the pooled results from the three colour genotypes (homozygous red, heterozygous red and homozygous wild type) of *O. niloticus*.

Since tilapias are multiple spawners, fecundity alone does not represent the best estimate of reproductive output, therefore spawning periodicity should also be considered. Overall mean ISI of *O. niloticus* was found to be 26.29±0.69 days similar to that reported for *O. niloticus* by Mires (1982), Rana (1986), for *O. mossambicus* by Rana (1986), for *O. aureus* by Lee (1979: cited in Rana, 1988) and for *T. zillii* by Coward and Bromage (1999). All the studies mentioned above used a similar method for estimating ISI i.e. by removing egg or fry. Although considerable variation in ISI was observed in all colour genotypes, the shortest spawning cycles observed were just 7 days and 6 days in homozygous red tilapia of no. 7 and homozygous wild type tilapia of no. 2, respectively, on one occasion. Siraj *et al.*, (1983) reported a 7 day spawning interval for *O. niloticus* while Coward (1997) observed 7 days and 6 days for two different strains of *T. zillii*.

Little is known of the factors governing spawning periodicity in tilapiines. The length of the ISI can be influenced by several factors. Water temperature has been suggested to play a crucial role for governing spawning periodicity (El-Zarka, 1962: cited in Coward, 1997). Since water temperature was controlled in the present study, it is unlikely that the observed variability in spawning periodicity of all colour genotypes of *O. niloticus* was caused by this. Lowe-McConnell (1982) reported that spawning capacity decreased with age in *O. macrocephalus*. Since fish used in this study were

approximately the same age, the variation in ISI was unlikely to be due to age differences. The number of spawnings per breeding season in the three-spined stickleback (*Gasterosteus aculeatus*) and the convict cichlid (*Cichlasoma nigrofasciatum*) is related to food ration (Wootton, 1990). This is unlikely to be the case in the present study since feeding was *ad libitum*. ISI can also be influenced by different types of social interactions especially visual, audible and chemical stimulation from conspecifics (Jalabert and Zohar, 1982). Although the females in the present study were not subject to direct conspecific male contact, possible chemical stimuli from males which were kept in settling tanks of the same systems where females were kept might have caused the shorter ISI. However, Marshall (1972), Silverman (1978 a,b), Coward (1997) reported that tilapias are able to spawn regularly even when isolated. Srisakultiew (1993) found that male *O. niloticus* separated from females by clear plastic partitions were capable of stimulating adjacent females via both chemical and visual stimuli. On the other hand, visual and chemical stimulus from adjacent males did not significantly alter spawning rates of *T. zillii* females in the same water body separated by plastic partitions (Coward, 1997). Coward and Bromage (1999) suggested that the variability of ISI in aquaria-held *T. zillii* might be influenced by visual and pheromonal stimuli from females showing asynchronous spawning cycles since the females were not subjected to conspecific male contact. The present study agreed with Tacon *et al.* (1996) suggesting that observed variability in ISI in aquaria-held *O. niloticus* was probably due to the individual's physiological responses to a variety of factors. Tacon *et al.* (1996) suggested that the history of the previous cycle might explain a part of the observed variability in ISI. They observed that a new batch of small early vitellogenic oocytes were present in the ovary before spawning in *O. niloticus*. Therefore some relationship are exist between the number of oocytes in developing batches and the time taken to complete vitellogenesis .

While there were a significant differences between the first month and last month of the experiment in terms of weight and length as expected, total fecundity and relative fecundity did not differ significantly over the 11 month period in the three colour genotypes of *O. niloticus* in the present study. Since the present study was based on the consecutive spawning of several females, not all the females contributed to all monthly variables (weight, length and fecundity), resulting in some of the variation in weight, length, total fecundity and relative fecundity in some months.

Total fecundity ranged between a minimum of 265 for heterozygous red female of no. 5 (198.4 g) and maximum of 1647 for the homozygous red wild type female of no. 3 (278.4 g) with a mean of 1096. Although fish size ranged widely over the time period, these figures generally compare with earlier studies which used hatchery reared *O. niloticus*, particularly with that of Rana (1986) who reported 1158 eggs per clutch for *O. niloticus* at the age of 2+ with a female weight range of 180-498 g. However the mean total fecundity of 1096 was lower than the mean total fecundity of 1546 given for hatchery reared *O. niloticus* with a size range of 190-397 g by Siraj *et al.* (1983) for the same age class. Myers and Hershberger (1991) reported that the mean total fecundity of strip-spawned *O. niloticus* (224.1 g) was 951. The mean total fecundity of 1096 is comparable with Philippine red tilapia (150-200 g) having 400-1200 eggs/spawn in the study of Galman and Avtalion (1983).

Significant relationships between total fecundity and fish size (weight and length) in homozygous wild type and in pooled data from all colour genotypes and between total fecundity and fish length in heterozygous red tilapia were also observed by Dadzie and Wangila (1980) in *T. zillii*, Blay (1981) in *S. galilaeus*, Siraj *et al.* (1983) and Rana (1986) in *O. niloticus* indicating that total fecundity was significantly related to fish size in teleosts. Rana (1988) reported that total fecundity is more closely associated with

maternal size than age in *O. niloticus*. On the other hand, no relationship was found between total fecundity and fish size in homozygous red tilapia. Although it is unclear as to why this so, higher sample size may have been needed to find stronger relationships between total fecundity and fish size in homozygous red *O. niloticus*. Similar findings were also observed in two strains of *T. zillii* namely strain "A" and "B" in that no relationship was found between fecundity and fish weight in strain "B" while significant relationships were observed between fecundity and fish size in strain "A" (Coward, 1997).

The mean relative fecundity of 4.44 is similar to that of 4.24 (Myers and Hershberger, 1991) but lower than 6.11 (Siraj *et al.*, 1983) and higher than 3.3 (Rana, 1986) in other studies on *O. niloticus*. Relative fecundity was found to be negatively related to fish size in all three colour genotypes and pooled data. This was also observed in *O. niloticus* and *O. mossambicus* (Rana, 1986) and in rainbow trout (Bromage *et al.*, 1992). This was attributed to an increase in egg size and the gradually diminishing rate of increase in fecundity with increasing fish size.

In the present study, egg size was investigated on 5 different females from each colour genotypes only once during experimental period in order to examine any differences between colour genotypes of *O. niloticus* in terms of egg size. Therefore, the sample size was very low for a good comparison to previous studies. The regression analysis found no relationship between egg weight, egg diameter, egg volume and fish size. Wootton (1973) found no evidence of a relationship between fish size and egg size in the three-spined stickleback. In tilapia species, no correlation between fish size and egg size in *T. zillii* varying from 20-120 g, in *T. tholloni* and *T. zillii* varying from 10-80 g, in *O. niloticus* and *O. mossambicus* within a narrow age class was found by Dadzie and Wangila (1980), Peters (1983) and Rana (1988), respectively.

Egg size in tilapias is generally species-specific (Lowe-McConnel, 1955: cited in Rana, 1986). Within each species however, egg size is generally larger in larger fish (Rana, 1988). Peters (1983) reported that in *O. niloticus* and *S. galilaeus*, that egg size increased with body size in smaller fish but plateaued long before fish reached their maximum size.

In tilapias, it is unclear as to whether maternal age or size is the predominant factor influencing egg size, though Rana (1988) reported that maternal age might be an important contributory factor. There is conflicting evidence on the influence of nutritional status of the female on egg size. For rainbow trout (Scott, 1962) and common carp (Hulata *et al.*, 1974), a reduced food intake lowered fecundity but did not significantly reduce egg or fry size, whereas Bagenal (1969) produced larger but fewer eggs under poor nutritional status in brown trout. On the other hand Townshend and Wootton (1984) reported that a low ration significantly reduced the egg diameter of the convict cichlid.

The effect of bigger egg size on the growth or survival of tilapia is unclear due to varying experimental conditions (Rana, 1988). Cridland (1962: cited in Rana, 1988) reported that egg size advances continued up to the end of the 90 days trial in *O. spilurus*. Siraj *et al* (1983) noted that the initial advantages of egg size on fry length were obscured. In *O. niloticus* and *O. mossambicus*, mean body length and dry body weight of emergent fry were not significantly related to egg size (Rana, 1988). No effect of egg size on growth and survival was reported for rainbow trout (Bromage *et al.*, 1990), Indian major carp, *Labeo rohita* (Sehgal and Toor, 1991) and pike, *Esox lucius* (Wright and Shoesmith, 1988).

Most studies of reproduction tend to consider fecundity and egg size as separate indicators of reproductive performance. It is generally accepted however, that there is an inverse relationship between fecundity and egg size; fish produce either more eggs of a

smaller size or fewer eggs of a larger size (Bromage *et al.*, 1992). Bromage *et al.* (1992) reported that 1000-3000 eggs/kg were produced in rainbow trout with a egg size of 4.5 mm in diameter. On the other hand, in multiple spawners such as *O. niloticus*, the total fecundity is greater than that of salmonid species. For example mean relative fecundity of *O. niloticus* in the present study is 4436 eggs/kg. Marine species such as cod, *Gadus marhua* and Atlantic halibut, *Hippoglossus hippoglossus* produce small pelagic eggs but tend to exhibit very high fecundities: several million eggs can be produced per season (Norberg, *et al.*, 1991). As a result of this "trade-off" it was suggested that total egg volume was a more appropriate index of egg production (Bromage *et al.*, 1992), since it considers the aggregate of both egg number and size. In this sense, no differences were found between the three colour genotypes of *O. niloticus* in terms of total egg volume and no relationship was observed between total egg volume and fish size. On the other hand, egg weight to body weight ratio was negatively correlated to fish size in pooled data from all colour genotypes ($P<0.001$). A similar correlation was reported for *T. zillii* (Coward, 1997) and for *O. niloticus* (Rana, 1986).

Little is known of how and why reproductive traits such as fecundity vary with consecutive spawns in tilapias. In the present study, the total fecundity, relative fecundity and ISI varied widely over consecutive spawns in all colour genotypes of *O. niloticus*. No relationships were found between ISI and fecundity in any single colour genotype or the pooled data. Only one homozygous red and one heterozygous red female showed a significant negative relationship between ISI and fecundity out of 34 tests. Lowe-McConnell (1982) reported that the number of eggs from an individual tilapia in equatorial lakes or ponds diminishes with each spawning. On the other hand, in laboratory-held fish, Lee (1979) found that there was a tendency for clutch size to increase with successive spawns in some individual *O. aureus*, *O. urolepis hornorum* and

O. niloticus. Siraj *et al.* (1983) and Mires (1982) also observed a trend for the number of eggs/clutch to increase with successive spawns for some individual *O. niloticus*. However, variability in fecundity of individual *O. niloticus* females during consecutive spawns was reported by Rana (1986), Mires (1983) and in *T.zillii* by Coward (1997).

In the present study, variation in ISI was also observed in all colour genotypes of tilapia. Overall C.V. in ISI ranged from 7.5% for a heterozygous female (no.1) to 78.1% for a homozygous wild type female (no.8.). Mires (1982) and Lee (1979) also reported significant variation in ISI for *O. niloticus*. Rana (1988) reported that ISI can be reduced by removing eggs from females after spawning. However, the same author indicated that in crowded aquarium conditions, ISI might be as long as those of natural breeding cycles in ponds even though eggs were removed from females.

The fecundity of partial or serial spawners may be modified by environmental factors (Wootton, 1982). Under natural conditions food supply played a critical role in egg size and fecundity observed over successive spawns in tilapia species (Lowe-McConnell, 1982). However, it is unlikely that variations in food supply were responsible for the observed variation in fecundity in laboratory-held tilapias. Pollution or abrupt changes in water quality and social environment effect the fecundity (Gerking, 1980). Although it is not clear why fecundity and ISI varied with successive spawns, the present study agreed with Coward and Bromage (1999) who suggested that fecundity and spawning interval may be controlled by complex inter-related mechanisms involving oocyte recruitment, growth and reproductive endocrinology.

In the present study, no pleitropic effects of red gene on survival rate *of O. niloticus* was found. Negative pleiotropic effects of body colour were reported for several fish species (see Tave, 1993). Wohlfarth and Moav (1970) found that both blue and gold common carp had lower growth rates than normal coloured common carp. Bondari

(1984) was also reported that albino channel catfish produced eggs that lower hatchability and poorer growth rate than normally pigmented channel catfish. A negative pleiotropic effect of red genotype on viabilities of red *O. aureus* and *O. niloticus* hybrid was reported by El-Gamal *et al.* (1988). Pruginin *et al.* (1988) observed that red hybrid fry of *O. mossambicus* X *O. niloticus* (known as Philippine red tilapia) were vigorously preyed upon by their parents and grey siblings and birds due to their conspicuous colouration.

The evidence from the present study shows that red colour phenotype does not appear to have any significant effect in the overall fecundity or viability of red tilapia compared to wild type fish from the same genetic background.

Chapter 5:

Production of YYRR neofemales and YYRR males in Nile tilapia, *O. niloticus*

5. Production of YYRR males and YYRR neofemales in Nile tilapia, *Oreochromis niloticus*

5.1. Introduction

5.1.1. Approaches to YY male production

Sexual dimorphism for commercial traits of aquacultural species is one of the primary reasons for interest in producing monosex populations. Although several methods have been proposed to control sex ratios, recent research has concentrated on the production of YY males in fish species with male heterogamety (e.g. *O. niloticus*) and ZZ females in fish species with female heterogamety (e.g. *O. aureus*) to produce genetically male fish populations by crossing YY males with homogametic XX females and homogametic ZZ males with ZZ neofemales, respectively, where the males has an advantage over the female. In fish species with male heterogamety where the females has an advantage over the male (e.g. Rainbow trout), genetically female fish populations can be produced by crossing XX neomales with homogametic XX females.

Yamamoto (1955) produced YY medaka for the first time using the technique of hormonal sex reversal and selective breeding. Yamamoto (1975) also succeeded in producing YY male goldfish which sired all male goldfish in the F1 generation when bred with normal XX females.

Production of YY supermale in the guppy, *Poecilia reticulata* was achieved with similar approaches described above by Kavumpurath and Pandian (1993). Embryos in gravid guppies were feminised by oral administration of estrogen and XY neofemales were identified by progeny testing. Crossing of XY neofemales with normal XY males

resulted in YY supermales which sired all-male offspring when crossed to normal XX females.

The first report of YY males in tilapia was reported by Varadaraj and Pandian (1989) and Scott *et al.* (1989). The supermale *O. mossambicus* were produced by combining the endocrine sex-reversal technique with crossing and gynogenesis. In their work, F1 generation was feminised by oral administration of DES (diethylstilbestrol) and XY neofemales were identified by progeny testing. The eggs from XY neofemales were fertilised with UV-irradiated sperm (254 nm for 10 minutes) and suppression of the second meiotic division was inhibited by heat shock at 41.1-42°C for 3-3.5 minutes commencing 2-5 min. after fertilisation. This produced diploid XX females and YY supermales.

The production of YY supermales and their potential for aquaculture in the Nile tilapia are described in the studies of Scott *et al.* (1989) and Mair *et al.* (1993, 1997, 1999). A supermale *O. niloticus* which consistently sired 100% male progeny when crossed to normal females was obtained by inducing meiotic gynogenesis in eggs of a natural sex-reversed XY female (Scott *et al.*, 1989). More recently, Mair *et al.* (1997) reported the results of a developed programme incorporating endocrine sex-reversal and controlled breeding techniques to produce YY supermale *O. niloticus*. In this breeding programme, XY neofemales were crossed to normal XY males to generate YY males in which YY males can be distinguished from normal males by progeny testing (Scott *et al.*, 1989; Mair and Little, 1991; Pandian and Varadaraj, 1990; Mair *et al.*, 1993, 1997). Details of this programme are described in Section 1.6. and illustrated in Figure 1.3.

YY male genotypes can also be produced by androgenesis (Thorgaard *et al.*, 1990; Parsons and Thorgaard, 1985; Scheerer *et al.*, 1991; Bongers *et al.*, 1994; Myers *et al.*, 1995a; Chapter 2 in this thesis). In different colour morphs of *O. niloticus* namely red, blond (Chapter 2 in this thesis) and wild type YY males (Myers *et al.*, 1995a) were obtained by crossing UV-irradiated eggs with normal sperm (XY). Diploidy was restored by suppression of the first mitotic division. The resulting male offspring were raised individually and sired all-male populations when crossed to normal XX females (Chapter 2 in this thesis).

Although gynogenesis and androgenesis can reduce the time-consuming procedure of progeny rearing and pedigree analysis of treated fish at sexual maturity for production of YY males, low survival rates with the potentially undesirable effects of inbreeding are major drawbacks to the use of these techniques. However, a few androgenetic or gynogenetic YY supermales may be sufficient for initiating large-scale YY male broodstock production by introgressive approaches.

5.1.2. Sex reversal

5.1.2.1.Hormonal sex-reversal of males to females

Large-scale production of YY male broodstock requires the efficient feminisation of sexually undifferentiated fry. The application of estrogens to feminisation of genotypic males to phenotypic females has been conducted on several fish species such as medaka (Yamamoto, 1955), rainbow trout (Goryczko *et al.*, 1991), channel catfish (Goudie *et al.*, 1983), coho salmon (Hunter *et al.*, 1982), guppy (Kavumpurath and

Pandian, 1992), fighting fish, *Betta splendens* (George *et al.*, 1994), zebra cichlid, *Cichlasoma nigrofasciatum* (George and Pandian, 1996), *O. mossambicus* (Varadaraj, 1989), *O. aureus* (Jensen and Shelton, 1979; Lahav, 1993; Melard, 1995) and *O. niloticus* (Mair *et al.*, 1993; Gilling *et al.*, 1993; Vera Cruz and Mair, 1994). Comparison of hormonal treatment protocols using estrogens for sex-reversal in tilapias are presented in Table 5.1.

5.1.2.2. Sex-reversal by temperature treatment

Studies have shown that the phenotypic sex in fish can be altered by environmental factors such as temperature (Conover and Kynard, 1981), pH (Beamish, 1993) and pollutants (Torblaa and Westman, 1980). The most commonly identified environmental variable inducing sex-change is temperature (Bull, 1983) which is known to be capable of overriding the genotypic sex determination in a small but increasing number of teleost fishes (Strüssmann and Patiño, 1995).

In fishes, thermal alteration of sex ratio (Temperature Sex Determination, TSD) has been reported especially among the atherinids. The most comprehensive study of TSD was in the Atlantic silverside (Conover and Kynard, 1981) in that low temperature favours the formation of females whereas high temperatures yield more males. Recently, thermolability of sex determination has been established under controlled thermal conditions using normal and putative monosex progeny of various fish species (Strüssman and Patiño, 1995). Sullivan and Schultz (1986) found that in the livebearing teleost fish, *Poeciliopsis lucida*, one strain produced almost all-male offspring at 30°C and female-biased sex ratios at 24°C while the other strain produced a 1:1 sex

Table 5. 1. Comparison of reported hormonal treatment protocols using estrogens for sex-reversal in tilapias. DES: diethylstilbestrol, EE: ethnylestradiol, ES: 17β-oestradiol, ME: methallibure, P: progesterone, F: flutamide, dph: days post hatching, DO: Dose, D: duration, d: days, h: hours

Species	Stages of fish treated	Hormone used	Treatment	% Female	Reference
O. mossambicus	7 dph	EE	DO: 50 $mgkg^{-1}$ in diet D: 19 d	100	Nakamura and Takahashi, 1973
O. mossambicus	6 dph	DES	DO: >100 $mgkg^{-1}$ in diet D: 11-15d	100	Varadaraj, 1989
O. mossambicus	first feeding fry	DES	DO: 10-125 $mgkg^{-1}$ in diet D: 14-34 d	98-100	Rosenstein and Hulata, 1993
O. mossambicus	first feeding fry	EE	DO: 50-100 $mgkg^{-1}$ in diet D: 14-32 d	100	Rosenstein and Hulata, 1993
O. mossambicus x *O. urelopis hornorum*	fertilised eggs	ES,P, F, and F+P	DO: 1-5000 μgl^{-1} in water bath D: 2 h-30 d	0	Rosenstein and Hulata, 1992
O. mossambicus x *O. urelopis hornorum*	first feeding fry	DES	DO: 125 $mgkg^{-1}$ in diet D: 33 d	98	Rosenstein and Hulata, 1993
O. mossambicus x *O. urelopis hornorum*	first feeding fry	EE	DO: 100 $mgkg^{-1}$ in diet D: 33 d	100	Rosenstein and Hulata, 1993
O. mossambicus	first feeding fry	EE	DO: 50-100 $mgkg^{-1}$ in diet D: 14-32 d	100	Rosenstein and Hulata, 1993

Table 5.1. Continued

Species	Stages of fish treated	Hormone used	Treatment	% Female	Reference
O. aureus	12 mm fry	EE+ME	DO: 100 $mgkg^{-1}$ each in diet D: 42 d	90	Hopkins *et al.*, 1979
O. aureus	12 mm fry	EE+ME	DO: 100 $mgkg^{-1}$ (EE) 10 $mgkg^{-1}$ (ME) in diet D: 42 d	66	Hopkins *et al.*, 1979
O. aureus	12 mm fry	DES	DO: 100 $mgkg^{-1}$ in diet D: 56 d	64	Hopkins *et al.*, 1979
O. aureus	first feeding fry	DES	DO: 100 $mgkg^{-1}$ in diet D: 23-30 d	100	Rosenstein and Hulata, 1993
O. aureus	first feeding fry	17α-EE+ME	DO: 100 $mgkg^{-1}$ each in diet D: 42 d	95	Mair *et al.*, 1987b
O. aureus	first feeding fry	17α-EE	DO: 100-200 $mgkg^{-1}$ in diet D: 40 d	94-98	Mélard, 1996
O. niloticus	9-12 mm fry	EE	DO: 100 or 200 $mgkg^{-1}$ in diet D: 25-59 d	62-78	Tayamen and Shelton, 1978
O. niloticus	9-12 mm fry	DES	DO: 100 $mgkg^{-1}$ in diet D: 25-59 d	88-90	Tayamen and Shelton, 1978

Table 5.1. Continued

Species	Stages of fish treated	Hormone used	Treatment	% Female	Reference
O. niloticus	first feeding fry	DES	DO: 100 $mgkg^{-1}$ in diet D: 10-25 d	89-98	Scott *et al.*, 1989
O. niloticus	8.7 mm fry	DES	DO: 400 $mgkg^{-1}$ in diet D: 28 d	80	Potts and Phells, 1993, 1995
O. niloticus	first feeding fry	DES	DO: 1000 $mgkg^{-1}$ in diet D: 10 d	78	Mair and Santiago, 1994 Vera Cruz and Mair, 1994
O. niloticus	first feeding fry	EE	DO: 100 μgl^{-1} in water bath D: 18 d	82-86	Gilling *et al.*, 1996

ratio at both temperatures. These authors suggested that in *P. lucida* populations sex ratio is influenced by both genetic and environmental factors. Strüssman *et al.* (1997) assessed the effect of temperature on progeny sex ratios in two other atherinid fish. In *Odontesthes bonariensis* all female progeny were produced at 17°C whereas fish exposed to about 25°C become male-biased. In contrast, in *Patagonina hatcheri* sex ratios are balanced (1:1) within the same range of temperature showing strong genotypic control. In channel catfish, the sex ratio was significantly skewed towards females in fish treated at 34°C whereas exposure to low and ambient temperatures did not effect the sex ratios (Patiño *et al.*, 1996). The effect of temperature on sex ratios of loach was reported by Arai *et al.* (1997). High temperature rearing with 25°C and 30°C for about 250 days after beginning of feeding gave significantly higher percentages of males compared to the sex ratios of fry reared at 20°C in normal crosses of XX females with XY males loach. Male progeny were observed at 25 and 30°C in gynogenetic all-female progenies of loach.

There have been several reports of temperature sex determination in tilapia (Desprez and Mélard, 1998; Mair *et al.* 1990; Baroiller *et al.*, 1995a, b; Baroiller, 1996). The sex ratio of *O. aureus* was reported to be skewed towards females by elevating rearing temperature (Yu and Lay, 1982: cited in Mair *et al.*, 1990) in mixed sex fry. On the contrary, Desprez and Mélard (1998) reported high male ratios at 34°C in *O. aureus*. In one out of seven experiment, 20% females were observed in putative all-male broods of *O. auresus* derived from neomales and subjected to 32°C (Mair *et al.*, 1990). In *O. mossambicus*, two cold temperature experiments at 19°C, yielded a significant excess of males (89% and 78%) compared to the control (Mair *et al.*, 1990).

The effect of temperature on sex ratios of *O. niloticus* was extensively studied by Baroiller *et al.* (1995 a,b). Treatments at 36°C in mixed sex fry of *O. niloticus* resulted in increased proportion of males (33-81%) (Baroiller *et al.*, 1995a). Baroiller *et al.* (1995b) reported that high temperatures (34-36°C) significantly increased the proportion of males in mixed sex families of *O. niloticus* (69-91%) and red tilapia (Florida strain) (98.3%). All female *O. niloticus* derived from the crosses of XX neomales with normal XX females were exposed to temperatures ≥32°C and the proportion of males increased from 0 to 91% as compared to the control (Baroiller, 1996). The author suggested that in *O. niloticus* sex was determined by genetic factors, temperature level and genotype-temperature interaction.

Abucay *et al.* (1999) exposed progeny of different genotypes (XX, XY and YY) of *O. niloticus* to high temperature (36.7±5°C) resulting in higher percentages of males being obtained in the putative all-female progeny whereas lower percentage of males were observed in all male (YY) progeny. The authors hypothesised that sex differentiation in YY males appears to be more labile than in normal males (XY).

Manipulation of water temperature may be a promising alternative option to hormones for control of sex in fishes because of consumer reaction to hormone-treated or chromosome-manipulated fish needed for the maintenance and periodical replenishment of sex-reversed broodstock (Patiño, 1997; Strüssmann and Patiño, 1995; Mélard, 1998). Although hormone sensitive periods of sex determination appear to have a timing and duration that is similar to the temperature sensitive period in *M. menida* (Conover and Fleisher, 1986), *O. bonariensis* (Strüssman *et al.*, 1996) and in *O. niloticus* (Baroiller *et al.*, 1995a, b), appropriate combinations of temperature and duration of thermal

manipulation will have to be established for reliable production of specific sex ratios (all-female or all-male) (Strüssmann and Patiño, 1995).

5.1.3. Viability of YY genotype

There are several published reports on the production of YY genotypes in fish. However, the viability of YY genotypes has been shown to be very low in some species. Yamamoto (1955) reported that the progeny sex ratios of some crosses of normal males with neofemales were 1:2.4 and 1:2.2 (Female: Male) instead of the theoretical 1:3 ratio and suggested reduced viability of YY males in medaka while other crosses gave a sex ratio of 1:3 indicating that in some progeny, YY males were viable (Yamamoto, 1963: cited in George *et al.*, 1994).

Further work by Yamamoto (1964: cited in Yamamoto, 1969) showed that crossing of orange-red X^rY^R neofemale *O. latipes* with normal orange-red X^rY^R or white X^rY^r males resulted in viable orange-red Y^RY^r and inviable Y^RY^R genotypes (R stands for xanthic pigmentation). A recessive lethal gene (ma) was also demonstrated in guppy located in the Y chromosome (Winge and Ditlevsen, 1938: cited in George *et al.*, 1994). YY males are viable only in the heterozygous (Y^{ma} Y^{pa}) condition.

Inviability of the YY genotype has been demonstrated in fighting fish (George *et al.*, 1994), zebra cichlid (George and Pandian, 1996), coho salmon (Hunter *et al.*, 1982) and rainbow trout (Parsons and Thorgaard, 1985). Kavumpurath and Pandian (1992) produced a very low number of YYmales in guppy (2 out of 21 males) and suggested that the viability of the YY genotype is a major problem for mass production of YY male

based broodstock. Evidently the survival of YY males is rare and that of YY neofemales is even rarer.

Viability of YY neofemales and YY males were demonstrated in *O. mossambicus* (Pandian and Varadaj, 1988 and 1990) and in *O. niloticus* (Scott *et al.*, 1989; Mair *et al.*, 1993, 1997; Tuan *et al.*, 1999). Several authors reported proportions of YY males not different from expected (25%) in progeny derived from XY neofemales and normal males and suggested that YY males have equal viability and fertility as normal males (Mair *et al.*, 1993).

5.1.4. Approaches to production of homozygous red YYRR males and YYRR neofemales

The combined use of hybridization and backcrossing, known as introgressive hybridization (Manwell and Baker, 1970: cited in Behrends and Smitherman, 1984) or the introductory cross (Kirpichnikov, 1981) is used to combine several valuable traits of closely related strains, species or even genera by plant and animal breeders. Therefore, it is theoretically possible to transfer any important traits from one strain of tilapia to another.

In Stirling red tilapia genetic analysis showed that red phenotype was caused by a dominant allele of an is an autosomal gene (RR or Rr): wild type is rr (McAndrew *et al.*, 1988). Crosses between homozygous red and wild type resulted in heterozygous red individuals exhibiting a range of black blotching from 0-24.6% of the skin surface. Although homozygous red individuals showed no blotching, a few isolated melanistic

spots were observed especially around the eyes and head region by McAndrew *et al.* (1988).

Both red colouration and YY genotype are valuable traits but the combination of both traits in pure *O. niloticus* lines would be of great interest to aquaculture. Production of homozygous red YYRR males and YYRR neofemales could be achieved by: (i) a breeding programme integrating genetic manipulation of sex determining mechanism suggested by Scott *et al.* (1989), Mair and Little (1991), Mair *et al.* (1993 and 1997) in a red *O. niloticus* strain. (The major drawback of this approach is the requirement of performing all the time consuming and the labour intensive steps.); (ii) introgressive hybridization approaches to combine red coloration and YY genotype by crossing existing wild type YYrr males and females from one strain or an isolate of a strain with a red strain. (iii) utilising existing homozygous red YYRR genotype by using both approaches described above for mass production. Details of these approaches are illustrated schematically in Fig. 5.1., 5.2., 5.3. and 5.4.

Production of genetically male tilapia (GMT) population by crossing of YY males from one strain with females of other strains of *O. niloticus* was reported by Capili (1995). Crosses of YY males of Egypt-Swansea strain with females of the Egypt-ICLARM, Ghana-ICLARM, Ghana-BFAR and FAC red tilapia strains produced variable sex ratios in the interstrain GMT produced, reported by Capili (1995). Tuan (1997) produced interstrain hybrid YY males by integrating YY males of the Egypt-Swansea strain into the Egypt-AIT strain. Both authors suggested that strain differences in sex determination in *O. niloticus* exist based on variation in sex ratios of the offspring.

5.1.5. Objectives

The present study was carried out to investigate the possibility of combining red coloration and YY genotype in *O. niloticus* by using several breeding techniques. The breeding schemes used in this study are presented in Fig. 5.1. (same as Fig 5.8.), Fig. 5.2. (same as Fig 5.9.) , Fig .5.3. (same as Fig 5.11.) and Fig. 5.4. (same as Fig. 5.14). The following approaches were investigated.

1- Evaluation of feminisation success by comparing hormonal and thermal feminisation on all YY male progeny of *O. niloticus*.

2- Production of YYRR males and YYRR neofemales by integrating YYrr males and YYrr neofemales from Egypt-Swansea-Philippines (ESP) isolate and red morph of Stirling (SR) isolate of Egyptian strain of *O. niloticus*.

3- Utilisation of existing androgenetic YYRR males of SR of *O. niloticus* to produce YYRR males and YYRR neofemales.

4- Further elucidation of the genetic bases of sex determination mechanism and colouration in *O. niloticus*.

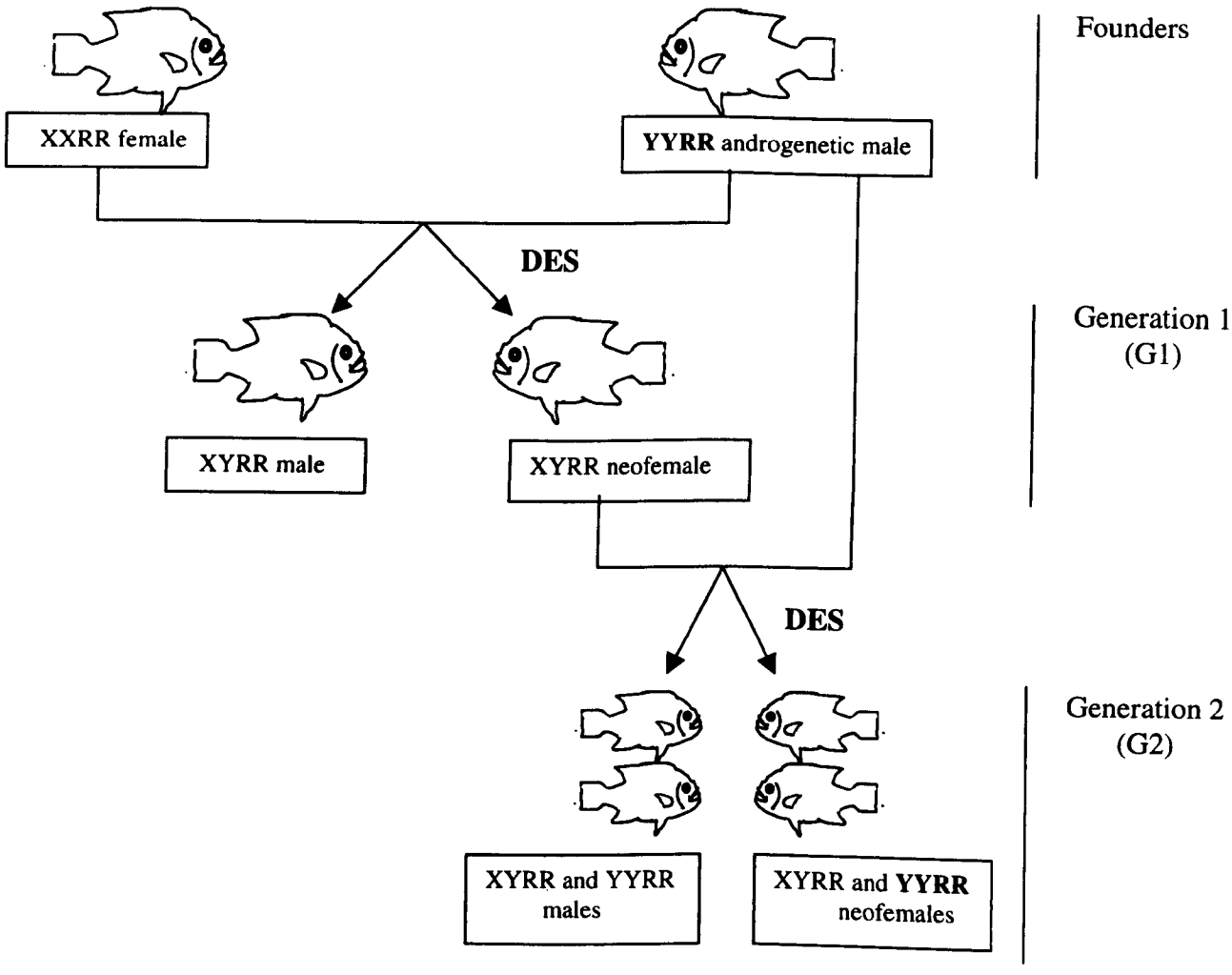


Figure 5.1. Schematic diagram of breeding scheme 1 for the production of pure-bred YYRR neofemales by using androgenetic YYRR male (Stirling red) of *O. niloticus* (same as Fig. 5.8.).

☐ Homozygous red (RR)

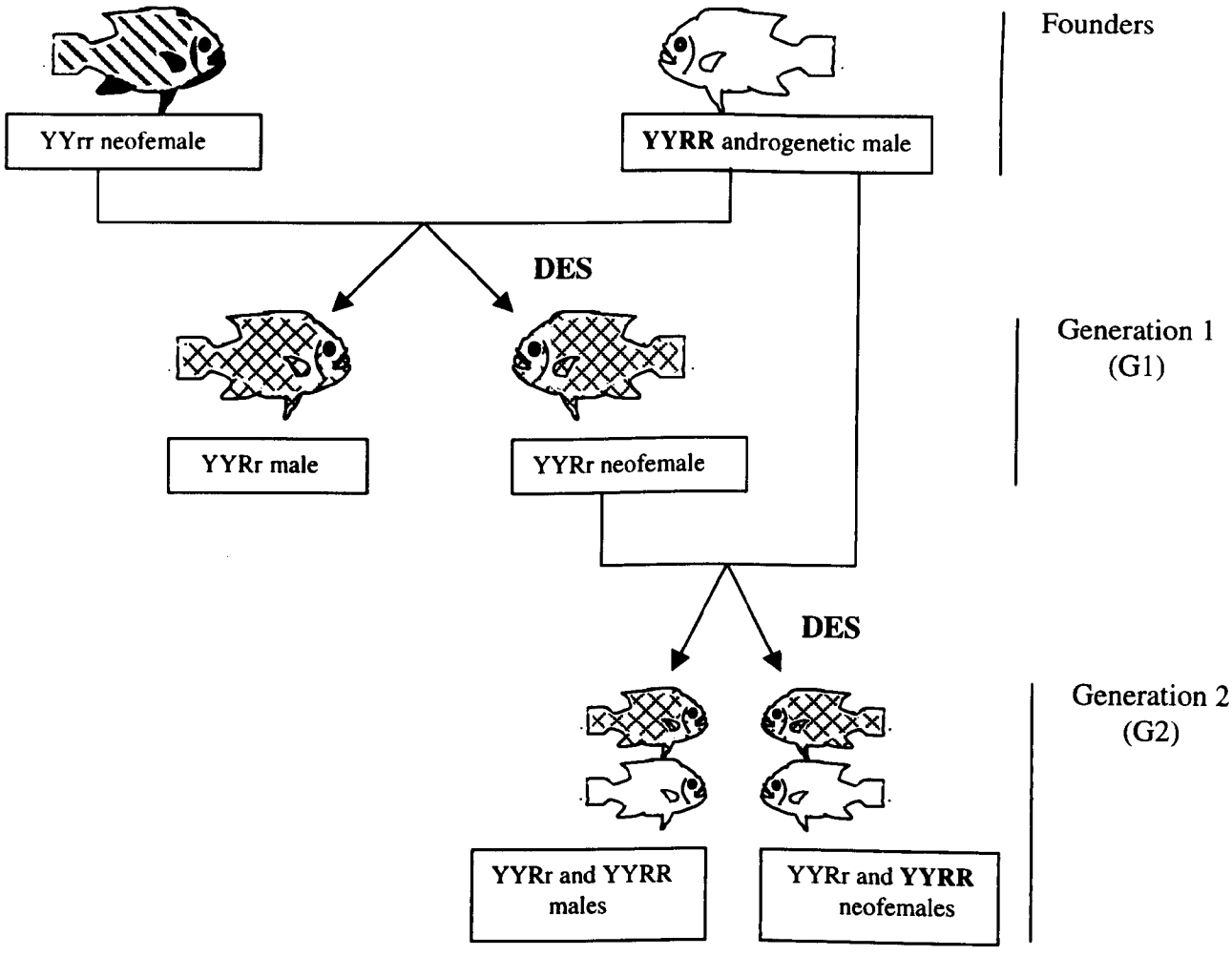


Figure 5.2. Schematic diagram of breeding scheme 2 for the production of YYRR males and YYRR neofemales by introgression of YYrr neofemales (Egypt-Swansea-Philippine) and androgenetic YYRR male (Stirling red) of *O. niloticus* (same as Fig. 5.9.).

Homozygous red (RR)

Wild type (rr)

Heterozygous red (Rr)

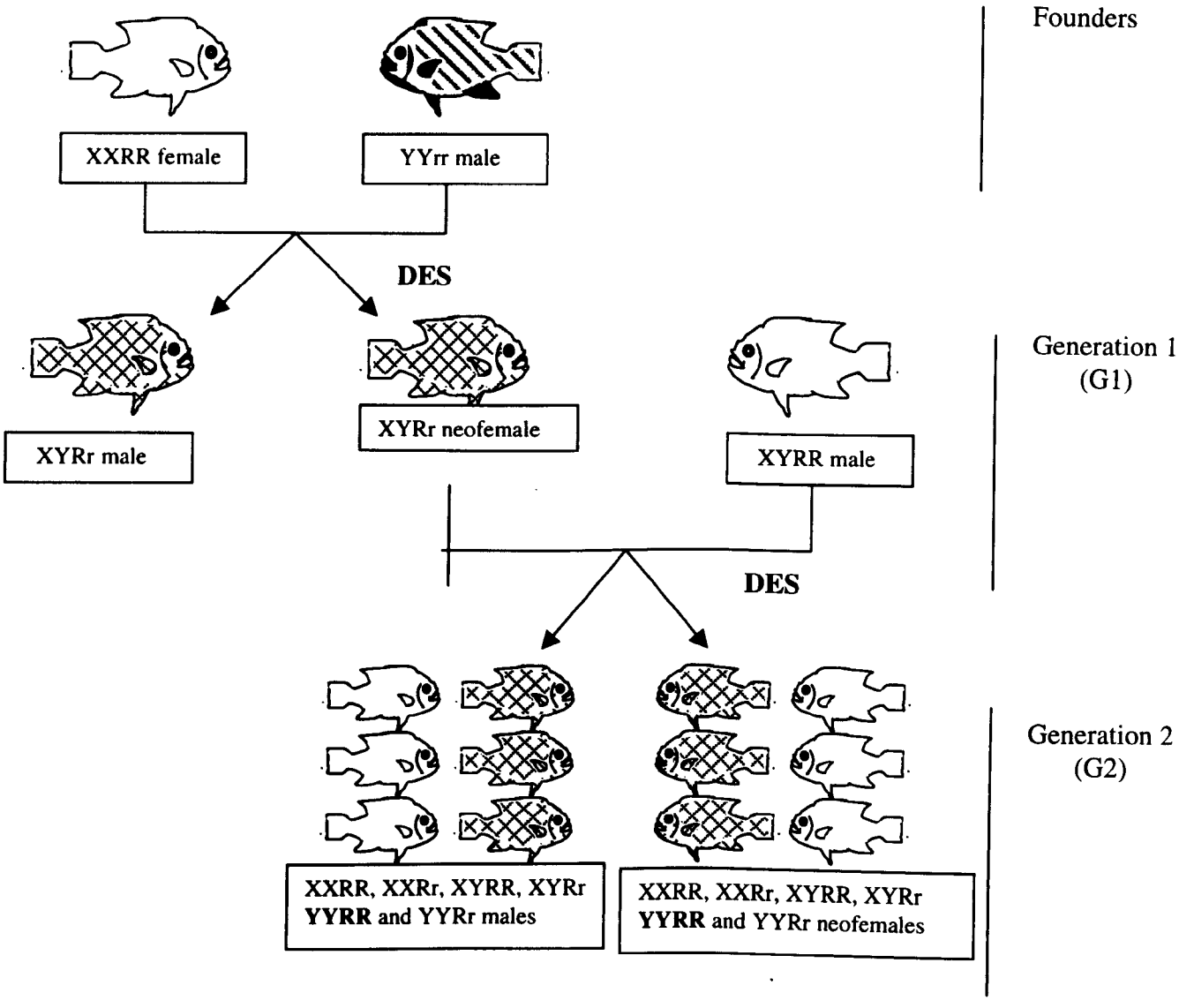


Figure 5.3. Schematic diagram of breeding scheme 3 for the production of YYRR males and YYRR neofemales by introgression of YYrr males (Egypt-Swansea-Philippine) and normal XXRR females (Stirling red) of *O. niloticus* (same as Fig. 5.11.).

Homozygous red (RR)

Wild type (rr)

Heterozygous red (Rr)

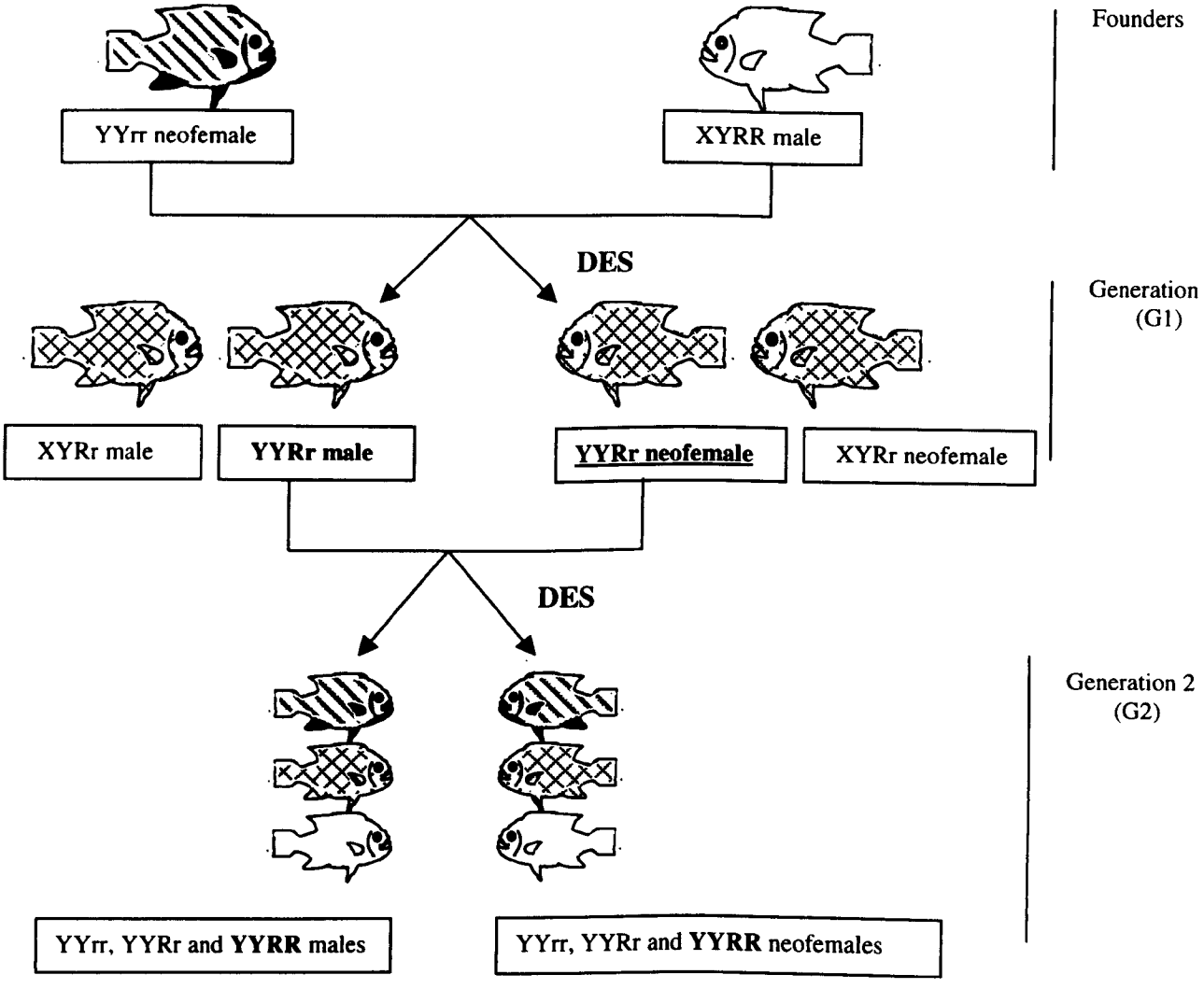


Figure 5.5. Schematic diagram of breeding scheme 4 for the production of YYRR males and YYRR neofemales by introgression of YYrr neofemales (Egypt-Swansea-Philippine) and normal XYRR male (Stirling red) of *O. niloticus* (same as Fig. 5.14.)

☐ Homozygous red (RR)

▨ Wild type (rr)

▩ Heterozygous red (Rr)

5.2. Materials and methods

5.2.1. Experimental strains of *O. niloticus*

The Egypt-Swansea-Philippine isolate (called ESP in this study) of Egyptian strain of *O. niloticus* used in this study originally came from Lake Manzala, Egypt and was transferred to the Institute of Aquaculture, University of Stirling in the late 1970's. Some fish from this stock were given to the University of Wales (UW), Swansea, in 1982.These were sent to Freshwater Aquaculture Center (FAC) of the Central Luzon State University (CLSU) in the Philippines in 1989 and 1990 (Tuan, 1997) and some were returned to UW in 1995. The experimental ESP wild type YYrr males and YYrr neofemales used in the present study were transferred to Institute of Aquaculture, Scotland via UW in 1995. According to Tuan (1997) ESP isolate of Egyptian strain of *O. niloticus* has low genetic diversity because (i) it was a small initial introduction from the Institute of Aquaculture (ii) a limited number of fish were spawned regularly to maintain stocks in Swansea; and (iii) the fish were subjected to genetic bottlenecks during transfers between Swansea and the Philippine. Plate 5.1. shows one of each the wild type YYrr males (A) and YYrr neofemales used in this study.

The origin of the red morph of Stirling isolate of Egyptian strain (called Stirling red, SR, in this study) *O. niloticus* is described by McAndrew *et al.* (1988). Two androgenetic SR YYRR males were also used in the present study (Plate 5.2.). One of them was produced by Myers *et al.* (1995a) and the other was generated from the experimental study described in Chapter 2.

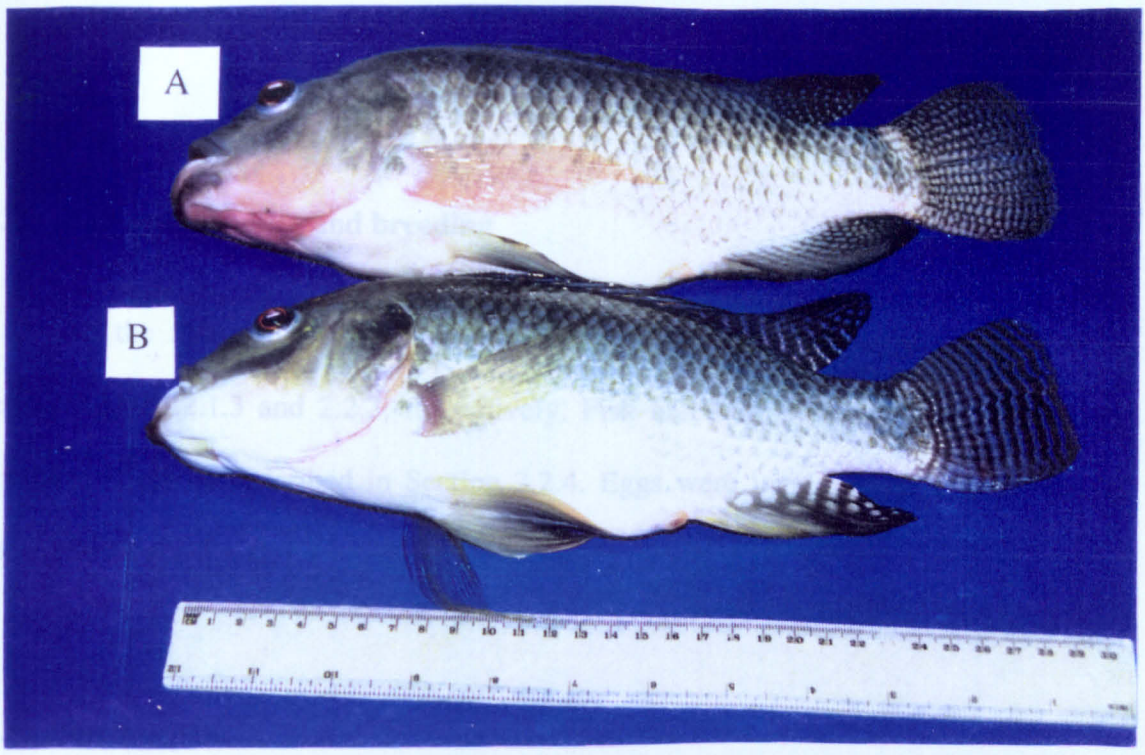


Plate 5.1. A YY male (A) and YY neofemale (B) of *O. niloticus*.

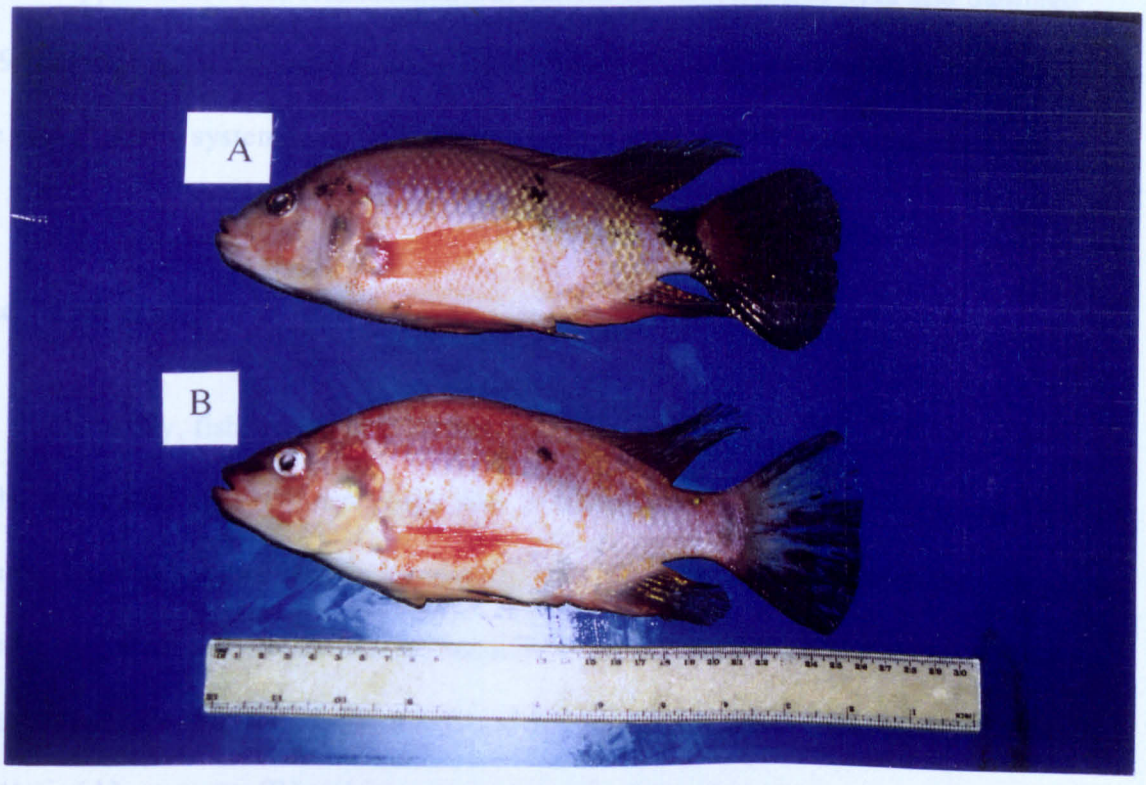


Plate 5.2. Androgenetic homozygous red YYRR male (013 296 291) (A) and 013 630 048 (B) of *O. niloticus*.

5.2.2. Fish maintenance and breeding

All the fish used in this study were maintained, fed and tagged according to Section 2.2.1, 2.2.1.3 and 2.2.3, respectively. Fish breeding, stripping and fertilisation were carried out as described in Section 2.2.4. Eggs were incubated in a recirculating system (Section 2.2.4.1)

5.2.3. Feminisation

Application of the feminisation was carried out according to Mair and Santiago (1994) by oral administration of diethylstilbestrol (DES) and described in section 2.2.7. The experimental system used for DES treatment was described in section 5.2.7.

5.2.4. Fish sexing

Generally, fish sexing was carried out as described in Section 2.2.8. Examination of the urogenital papilla morphology was performed on mature fish at the age of 4-6 months. However, when undeveloped urogenital papillae were observed, the fish were killed and gonads were removed for accurate examination since the morphology of the urogenital papilla may not be clear in hormone treated fish. Plate 5.3 shows the structure of a testis (A), an ovary (B) and intersex gonad (C) of *O. niloticus*.

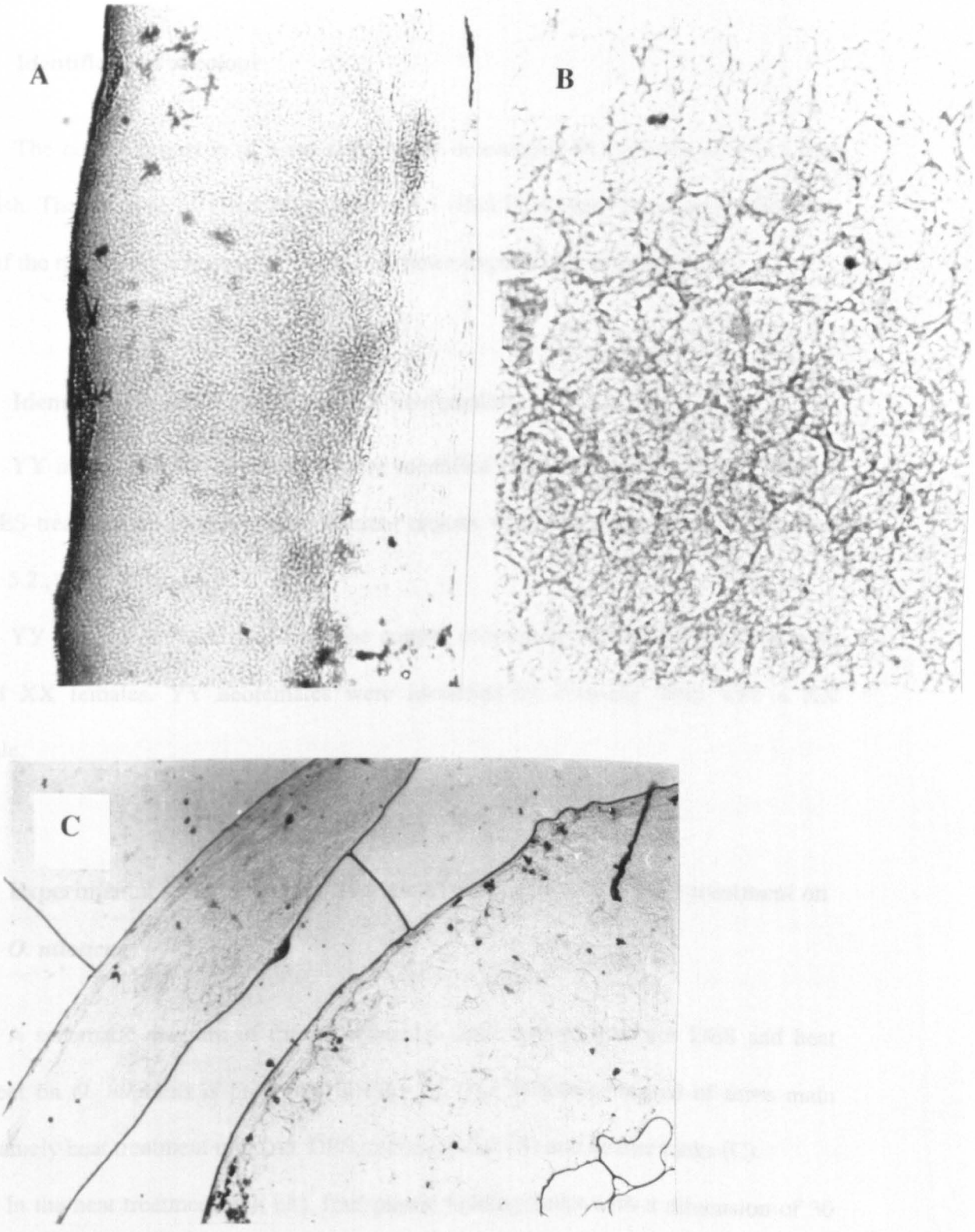


Plate 5.3. Photomicrographs of gonads of (A) male (X 10), (B) female (X 40) and (C) intersex (X 4) *O. niloticus* at 12 weeks. The intersex (C) shows one gonad has ovarian characteristics and the other testicular characteristics.

5.2.5. Identification of colour

The colour genotype of a red fish can be determined by crossing it with a wild type fish. The ratios of 1:0 (Red:Wild type) or 1:1 (Red:Wild type) are expected from this cross if the red parent is homozygous (RR) or heterozygous (Rr), respectively.

5.2.6. Identification of YY males and YY neofemales

YY males and YY neofemales were identified from two groups of fish (control and DES-treated, respectively) from different crosses which are schematically described in Fig. 5.2., 5.5., 5.8. and 5.9.

YY males were identified from the control crosses through progeny testing with normal XX females. YY neofemales were identified by crossing them with a XX neomale.

5.2.7. Experimental system used for diethylstilbestrol (DES) and heat treatment on *O. niloticus*

A schematic diagram of the experimental static system used for DES and heat treatment on *O. niloticus* is presented in Fig. 5.1. The system consisted of three main units namely heat treatment unit (A), DES treatment unit (B) and header tanks (C).

In the heat treatment unit (A), four plastic holding tanks with a dimension of 30 cm X 19 cm X 17 cm (A1), supported by bricks from underneath were placed into a glass aquarium with a dimension of 120 cm X 40 cm X 40 cm (A3). The whole system was

filled with clean and aerated tap water before the experiment started. The desired temperature was maintained using a 0-100°C range thermostatic heater with a stirrer pump (A2) (Gallenkamp, Thermo stirrer 85, 220-240V, EEC) placed in the glass holding tank (A3). The stirrer pump provided the circulation of water around the plastic holding tanks (A1).

The DES treatment unit (B) contained six plastic holding tanks with a dimension of 30 cm X 19 cm X 17 cm (B1). A thermostatic heater (Visi-Therm, UK.) having a range of 18-32°C was placed in each tank. Three of them were used for control group and three for DES treatment. The water temperature was maintained at 28±1°C during the experimental period.

Two header tanks formed the header tanks unit (C). A plastic header tank with a dimension of 60 cm X 44 cm X 41 cm (C2) used for topping up the DES treatment tanks (B) and a thermostatic heater (Visi-Therm, UK.) having a range of 18-32°C was placed in it to maintain the water temperature at 28±1°C. The water temperature in the glass header tank with a dimension of 62 cm X 30 cm X 30 cm (C1) was maintained at 32°C with a thermostatic heater (Visi-Therm, UK.) having a range of 18-32°C and adjusted to 36°C by adding boiled tap water before filling the plastic holding tanks (A1) in the heat treatment unit (A).

Aeration was provided to each plastic holding and header tank through an air pump during the experimental period. All plastic holding tanks were cleaned by siphoning the excess food and water and water were then topped up with clean aerated

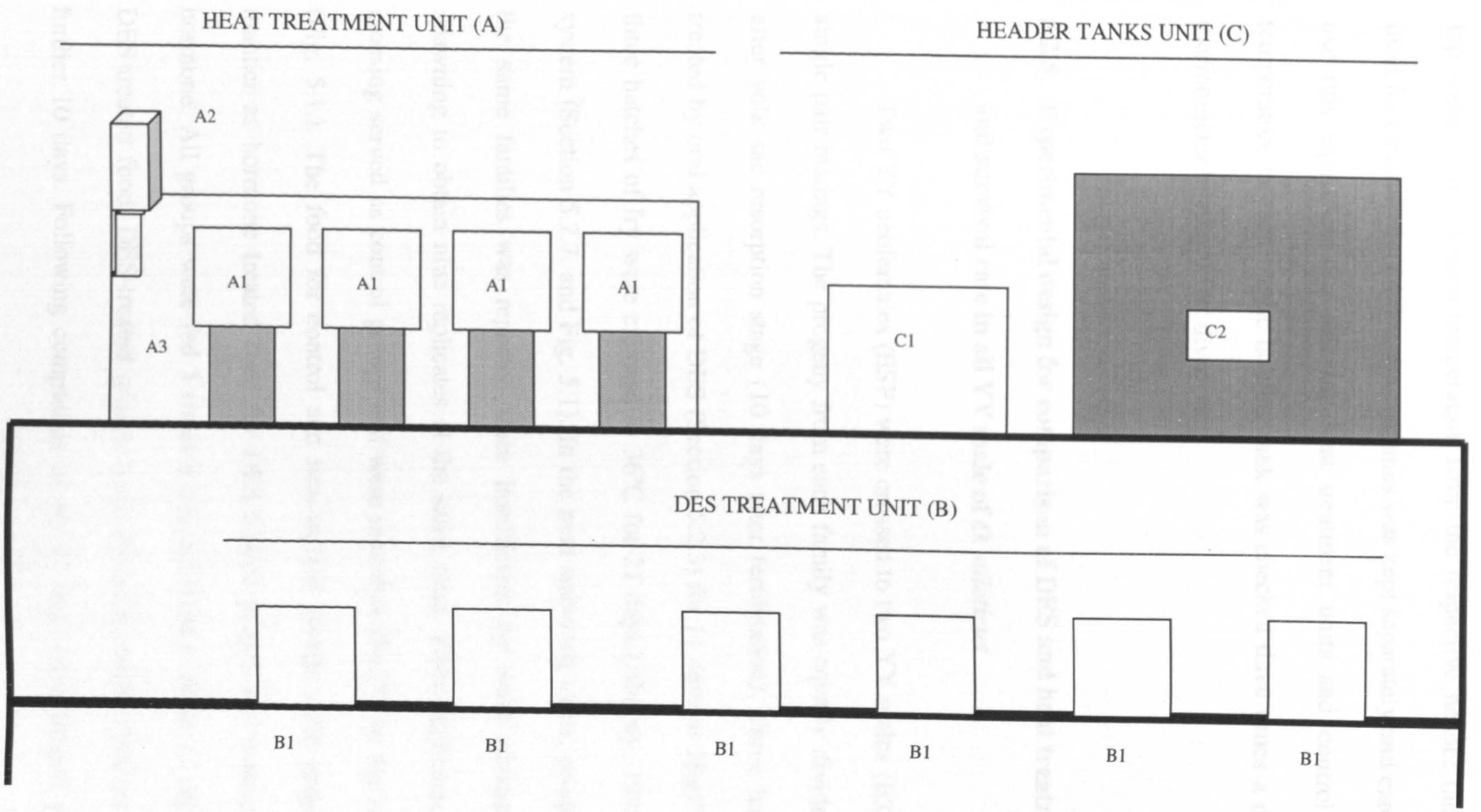


Figure 5. 5. Schemetic diagram of experimental static system used for DES and heat treatment on *O. niloticus*. (A: Heat treatment unit, A1: Plastic holding tanks, A2: Thermostatic heater, A3: Glass holding tank, B: DES treatment unit, B1: Plastic holding tanks, C: Header tank unit for replacement of water in fish holding tanks, C1: Glass header tank, C2: Plastic header tank.

tap water at the desired temperature from the respective header tanks. The equipment used for cleaning the DES treatment tanks was kept separately and care was taken not to use this equipment for cleaning heat treatment units and control tanks. The water temperature in each plastic holding tank was checked three times a day with a mercury thermometer having 0.1°C division.

5.2.8. Experimental design for comparison of DES and heat treatment on sex ratio and survival rate in all YY male of *O. niloticus*

Two YY neofemales (ESP) were crossed to two YY males (ESP) to generate two single pair matings. The progeny from each family was equally divided into six batches after yolk sac resorption stage (10 days after fertilisation). Three batches of fry were treated by oral application of DES (Section 5.2.3) for 11 days at 28±1°C, while the other three batches of fry were exposed to 36°C for 21 days (Abucay, 1999) in a static tank system (Section 5.2.7. and Fig. 5.1). In the next spawning cycle, production of fry from the same families was repeated since insufficient fry were obtained from the first spawning to obtain nine replicates at the same time. Three replicates from the second crossing served as control groups and were reared at 28±1°C in the same static system (Fig. 5.1.). The food for control and heat-treated groups were prepared in a similar manner as hormone treated food for DES-treated groups but without the addition of hormone. All groups were fed 3 times a day *ad libitum*. After 11 days treatment with DES-treated food, DES-treated groups were fed with control food as other groups for further 10 days. Following completion of the 21 days experimental period, all groups

were transferred to the early fry rearing system (Section 2.2.1.1.1) and reared for sexing by gonad squash method as described by Guerrero and Shelton (1974) (Section 5.2.4.).

5.2.9. Statistical analysis

For comparison of DES and heat treatment on sex ratio and survival rate in all YY male *O. niloticus*, heterogeneity chi-square tests were used between replicates of experimental groups. Intersex individuals were not used in calculating χ^2 for sex ratios. Since there was no heterogeneity detected between experimental groups in terms of survival rate and/or sex ratio data from each replicate of experimental group, analyses of variance or non-parametric Kruskal-Wallis test were applied on arcsine transformed percentages of survival rate and sex ratio according to the distribution of data to test for differences between experimental groups.

In the experiments on production of YYRR males and YYRR neofemales by different breeding programmes, the success of feminisation was assessed by comparing the sex ratios of DES-treatment groups to their respective control using a 2x2 chi-squared contingency test. The sex ratios of all control groups were tested against 1 male:1 female (or 1 male:3 female in the cross of XY x XY) sex ratio using a chi-squared test or Fisher exact test where the expected frequency is less than 5. The sex and colour genotype of YY males and YY neofemales were identified through progeny testing with normal wild type female (XXrr) and neomale (XXrr), respectively (Section 5.2.5 and 5.2.6).

A statistical criterion used for the designation of YY males and YY neofemales was adopted from Mair *et al.*, (1997). Sex ratios produced by crosses of YY genotypes with XX genotypes were tested against 1:1 ratio using a chi-squared test. YY males and YY neofemales were denoted as those producing almost or completely all-male progeny at a probability level of 0.1% ($P<0.001$) in respective progeny testing. When progeny sex ratio of a genotype was different from 1:1 at $P<0.01$ but not at $P<0.001$, no classification of genotype was assigned. Chi-squared heterogeneity values were calculated to assess the homogeneity of progeny sex ratios obtained from the same crosses of genotypes.

For comparison of feminisation rates between different crosses of different genotypes, the Kruskal-Wallis test was applied on arc-sine transformed data since data were not normally distributed. Results were presented as not-transformed data on the figure.

5.3. Results

5.3.1. Comparison of DES and heat treatment on sex ratio and survival rate in all YY male *O. niloticus*

Results obtained from control, DES and heat treated all YY male *O. niloticus* are shown in Table 5.2. and mean percentage values of survival rate, female, male and intersex are graphically presented in Fig. 5.6. A, B, C and D, respectively. The highest mean survival rates of 97.5±0.85% and 97.3±0.82% were obtained in control and DES treated groups which were significantly higher than the mean survival rate of 62.9±9.82% in heat treated groups ($P<0.05$).

Only males were observed in the control groups, while mean percentages of 33.8±1.50% and 32.0±5.21% female were produced in DES and heat-treated groups, respectively, indicating that feminization had taken place. The mean female percentages in the DES and heat treatment groups were not significantly different ($P>0.05$). Although no intersex gonads were observed in any control groups, mean values of 1.6±0.8% and 18.5±2.45% intersex progenies were produced in DES and heat-treated groups, respectively. The mean intersex percentages in heat treatment groups were significantly higher than the mean intersex percentages in DES treatment groups ($P<0.05$).

5.3.2. Production of pure-bred YYRR neofemales by using androgenetic YYRR male (Stirling red) in *O. niloticus*

The experimental design used to produce pure-bred YYRR neofemales by using an androgenetic YYRR male (SR) is schematically presented in Fig. 5.8, page 229).

Table 5.2. Results obtained from control, DES and heat treatment of monosex (YY) *O. niloticus* derived from two single pair matings. (INO: Number of fry at start of treatment, ENO: Number of fry at end of treatment, S: Survival rate, SR: Sex ratio as female: male: intersex, F: Female, M: Male, I: Intersex. SE: Standard error). Common superscript in the same row is not significantly different.

	Experiment I Control Replicate No			Experiment II Control Replicate No			Experiment I DES Treatment Replicate No			Experiment II DES Treatment Replicate No			Experiment I Heat Treatment Replicate No			Experiment II Heat Treatment Replicate No		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
INO	73	73	73	58	58	58	73	73	73	58	58	58	73	73	73	59	59	59
ENO	71	73	73	56	55	56	70	69	72	56	58	57	64	65	49	16	36	27
% S	97.3	100	100	96.5	94.8	96.5	95.9	94.5	98.6	96.5	100	98.3	87.7	89	67.1	27.1	61	45.5
SR	0:30:0	0:30:0	0:58:0	0:43:0	0:22:0	0:33:0	19:41:0	18:40:0	16:25:0	13:24:1	13:27:2	18:29:1	23:21:9	20:20:4	11:22:7	2:1:2	4:12:4	2:10:1
%F	0.0	0.0	0.0	0.0	0.0	0.0	31.6	30.0	39.0	34.2	30.9	37.5	43.4	45.4	27.5	40.0	20.0	15.4
% M	100.0	100.0	100.0	100.0	100.0	100.0	68.4	70.0	61.0	63.2	64.3	60.4	39.6	45.5	55.0	20.0	60.0	76.9
%I	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.6	4.8	2.1	17.0	9.1	17.5	40.0	20.0	7.7

Mean %S ± SE	97.5±0.85[b]	97.3±0.82[b]	62.9±9.82[a]
Mean %F ± SE	0.0±0.00	33.8±1.50[a]	32.0±5.21[a]
Mean %M ±SE	100.0±0.00[b]	64.6±1.59[a]	49.5±7.91[a]
Mean % I ± SE	0.0±0.00	1.6±0.80[a]	18.5±2.45[b]

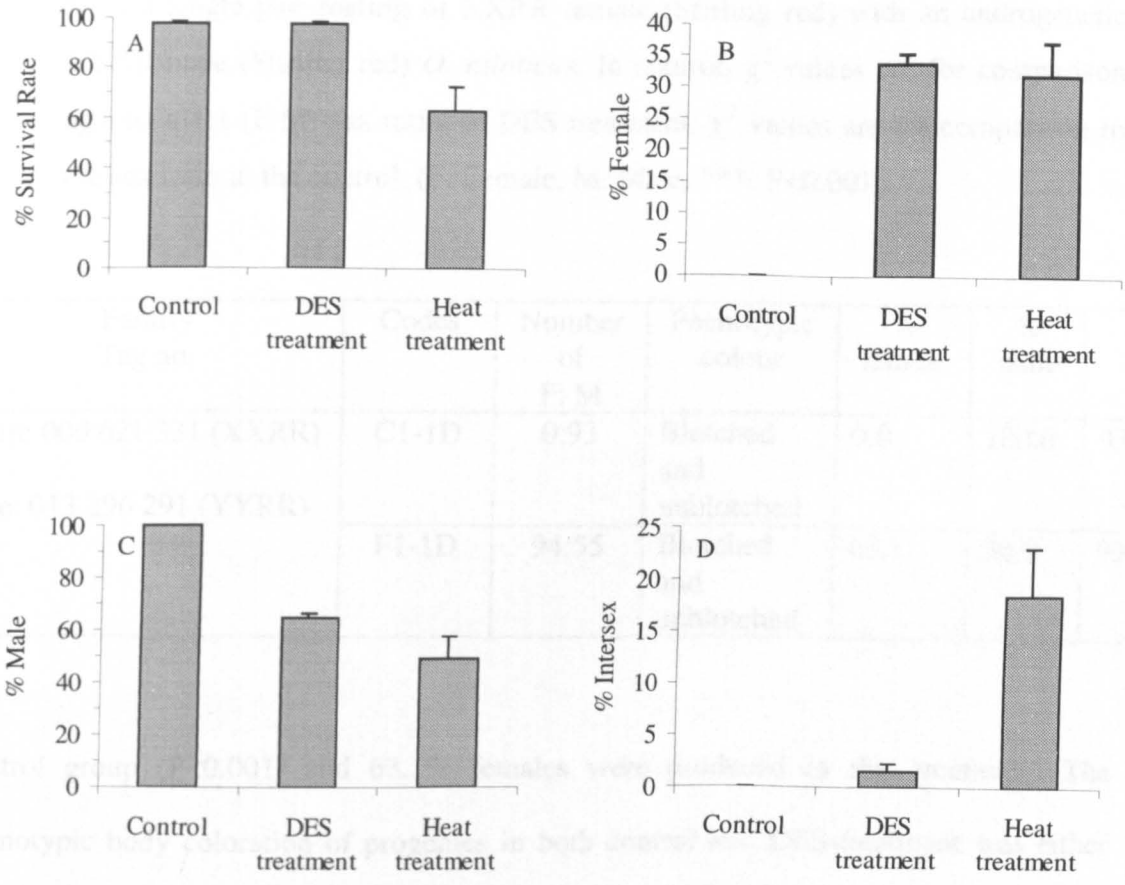


Figure 5.6. Mean values (%) of survival rate (A), females (B), males (C) and intersexes (D) in control, DES and heat-treated YY male *O. niloticus*.

The sex ratios in control and DES-treated groups of (Generation 1) G1 progenies derived from a pure-bred single pair mating of normal XXRR female (SR) with an androgenetic YYRR (SR) to produce pure strain XYRR neofemales are presented in Table 5.3. In the control group (C1-1D), 100% male progenies were produced as expected in this cross and these were significantly different from 1:1 ($P<0.001$). The sex ratio of the DES-treated group (F1-1D) was significantly different from that of the

Table 5.3. Sex ratios in control (C1-1D) and DES-treated (F1-1D) G1 progeny derived from a single pair mating of XXRR female (Stirling red) with an androgenetic YYRR male (Stirling red) *O. niloticus*. In control, χ^2 values are for comparison against a 1:1 (F:M) sex ratio; in DES treatment, χ^2 values are for comparison to the sex ratio in the control. (F: Female, M: Male, ***: P<0.001).

Family Tag no.	Codes	Number of F: M	Phenotypic colour	% female	% male	$\chi^2_{(1)}$
Dam: 000 621 331 (XXRR) Sire: 013 296 291 (YYRR)	C1-1D	0:93	Blotched and unblotched	0.0	100.0	93.00***
	F1-1D	94:55	Blotched and unblotched	63.1	36.9	93.29***

control group (P<0.001) and 63.1% females were produced in this treatment. The phenotypic body coloration of progenies in both control and DES-treatment was either pure red or black blotched (from slight to heavy).

The sex ratios of (Generation 2) G2 progenies (control and DES-treatments) derived from crossing of two XYRR neofemales (SR) with either the original androgenetic father or another androgenetic YYRR male are presented in Table 5.4. In the control groups, 100% male progenies were produced and the sex ratios were significantly different from a 1:1 ratio (at P<0.001 for C2-1D and C2-2D). The proportion of females in the DES-treated groups ranged between 92.2%-93.2%, which was significantly different from their respective controls (P<0.001). The progenies of all groups showed pure red colouration or blotching on their body surface.

Table 5.4. Sex ratios in control (C2-1D and C2-2D) and DES-treated (F2-1D and F2-2D) G2 progeny derived from two single pair matings between G1 XYRR neofemales and androgenetic YYRR *O. niloticus* males (Stirling red). In controls, χ^2 values are for comparison against a 1:1 (F: M) sex ratio; in DES treatments, χ^2 values are for comparisons to the sex ratio in the respective controls. (F: Female, M: Male, **: P<0.01, ***: P<0.001)

Families Tag no.	Codes	No. of blotched red F:M	No. of unblotched red F:M	% female	% male	$\chi^2_{(1)}$
Dam: 012 622 881 (XYRR)	C2-1D	0:26	0:2	0.0	100.0	28.00***
Sire: 013 296 291 (YYRR)	F2-1D	27:0	41:5	93.2	6.8	75.65***
Dam: 008 823 263 (XYRR)	C2-2D	0:0	0:13	0.0	100.0	13.00***
Sire: 013 630 048 (YYRR)	F2-2D	30:1	29:4	92.2	7.8	46.25***

Eight neofemales from G2 crosses (between XYRR neofemales with androgenetic YYRR males) were tested and four were identified as XYRR (Table 5.5.). The percentages of males in progeny of these XYRR neofemales ranged between 35.0-55.0% with a mean of 46.8%. Two neofemales from this group were classified as YYRR (Table 5.5.) which was not significantly lower than the expected number of YYRR genotypes from these crosses (see Table 5.6.). Of the eight neofemales tested, two were not classified because their progeny sex ratios were different from 1:1 ratio at P<0.01 but not at P<0.001 (Table 5.5.). The progeny test results of these G2 neofemales are summarised in Table 5.6.

Table 5.5. Results of progeny testing to identify YYRR *O. niloticus* neofemales from DES-treated G2 progeny (from G1 XYRR neofemale x androgenetic YYRR male crossses, see Fig. 5.8.). (F: Female, M: Male, ??: Unclassified, *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$, for original family of fish see Table 5.4.)

XY/YY RR females tag no. (Original family)	No. of fry sexed	No. of M:F	% male	χ^2 (1) (1:1)	Predicted genotypes
00-013C-B170 (F2-1D)	36	16:20	44.4	0.44	XY
00-013D-F70B (F2-1D)	36	29:7	81.0	13.44***	YY
00-013E-10FE (F2-1D)	37	27:10	73.0	7.81**	??
00-013C-B44D (F2-1D)	40	14:26	35.0	3.60	XY
00-013E-135E (F2-1D)	36	19:17	52.8	0.11	XY
00-013E-4986 (F2-2D)	61	53:8	86.9	33.20***	YY
00-013E-58C9 (F2-2D)	30	23:7	76.7	8.53**	??
00-013E-3EE6 (F2-2D)	20	11:9	55.0	0.20	XY

Table 5.6. Summary of results of progeny testing to identify YYRR *O. niloticus* neofemales from DES-treated G2 progeny (from G1 XYRR neofemale x androgenetic YYRR male crossses, see Fig. 5.8.).

Description	**Females**
No. of families from which fish tested	2
No. of fish tested	8
No. of fry sexed	296
Average family size	37
No. of genotypes classified as XYRR	4
No. of genotypes classified as YYRR	2
Expected no. of YYRR genotypes (50%)	4
Exact probability for binomial test	0.3
No. of fish not classified	2
Male percentage in progeny from XYRR genotype	35.0-55.0
Mean male percentage in progeny from XYRR genotype	46.8
Male percentage in progeny from YYRR genotype	81.0-86.9
Mean male percentage in progeny from YYRR genotype	84.0

Unexpected colour segregation was obtained from the crosses of G2 neofemales with the XXrr neomale. These results are presented in Table 5.7. The phenotypic colouration of progenies derived from crosses of six XYRR or YYRR neofemales with an XXrr neomale segregated into red and wild type. Comparison of the observed colouration ratios of progenies with a 1:1 (Red:Wild type) ratio showed no significant differences (P>0.05).

Fig 5.7. shows the frequency distribution of sex ratios in progeny testing of DES-treated G2 *O. niloticus* neofemales (from G1 XYRR neofemale x androgenetic YYRR male crosses).

Table 5.7. Colour segregation in offspring of G2 females in breeding scheme 1 (see Fig. 5.8.). Females were crossed to an XXrr neomale. χ^2 values are for comparison against a 1:1 ratio. (Red:Wild type). (n.s.: not significant, for original family see Table 5.4.)

Females no. (Original family)	Presumed genotype	Offspring		
		Observed number		χ^2 $_{(1:1)}$
		Red	Wild type	
1 (F2-2D)	RR	41	51	1.09 n.s.
2 (F2-2D)	RR	105	107	0.02 n.s.
3 (F2-2D)	RR	62	63	0.01 n.s.
4 (F2-2D)	RR	66	84	2.16 n.s.
5 (F2-2D)	RR	135	161	2.28 n.s.
1 (F2-1D)	RR	26	24	0.08 n.s.

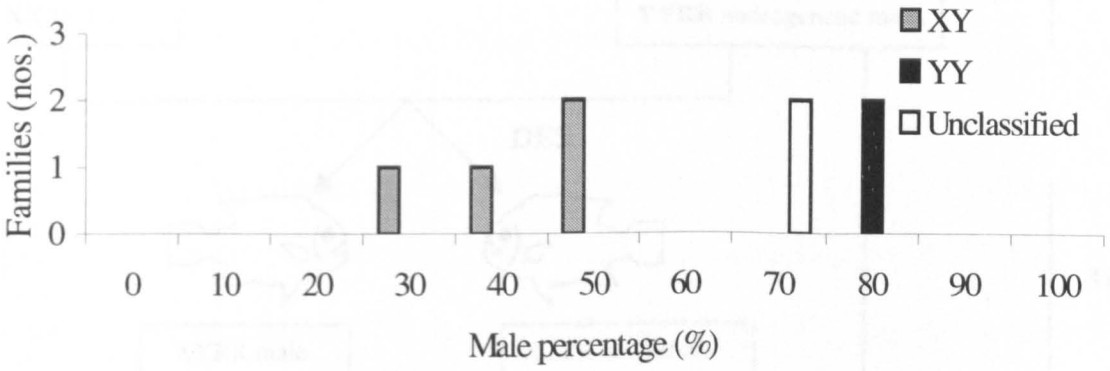


Figure 5.7. Frequency distribution of sex ratios in progeny testing of DES-treated G2 *O. niloticus* neofemales (from G1 XYRR neofemale x androgenetic YYRR male cross). All females were crossed to an XXrr neomale.

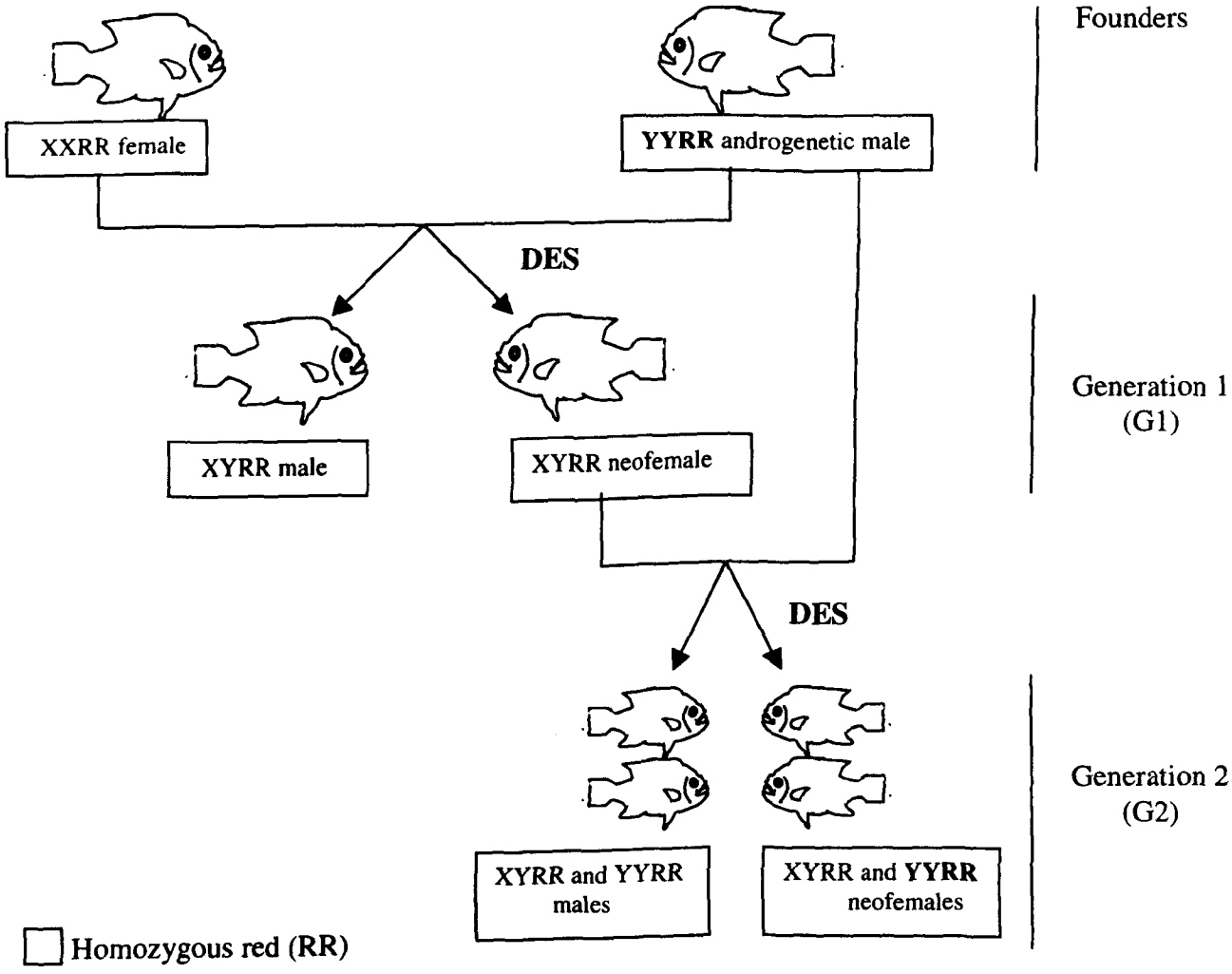


Figure 5.8. Schematic diagram of breeding scheme 1 for the production of pure-bred YYRR neofemales by using androgenetic YYRR male (Stirling red) of *O. niloticus*.

YYRR neofemales were produced using the breeding scheme 1 described above. Progenies from G1 and G2 crosses derived from XXRR x YYRR and XYRR x YYRR, respectively, were successfully feminized using DES treatment. This breeding scheme took 2 ½ generations and low numbers of pure-bred YYRR neofemales (only 2) were produced. The colour segregation of 1:1 (red: wild type) was obtained from the crosses between XYRR or YYRR neofemales with an XXrr neomale which was not predicted from these crosses.

5.3.3. Production of YYRR males and YYRR neofemales by introgression of YYrr neofemales (Egypt-Swansea-Philippine) and androgenetic YYRR males (Stirling red) in *O. niloticus*

The experimental design used to produce YYRR males and YYRR neofemales by introgression of a YYrr neofemale (ESP) and an androgenetic YYRR (SR) is schematically presented in Fig. 5.9. (page 233). The sex ratios of G1 progenies in control and DES-treatment groups derived from a single pair mating between a YYrr neofemale (ESP) and an androgenetic YYRR male (SR) are shown in Table 5.8. In the control group, the sex ratio was 100% male and significantly different from 1:1 ($P<0.001$). A female percentage of 44.8% was achieved in the DES-treated group, which significantly differed from the control group ($P<0.001$). The phenotype of progenies in both control and DES-treated groups was red as expected from this cross.

In G2 crosses, one of the YYRr neofemale offspring was backcrossed to its father (SR) while the other one was crossed to another different androgenetic YYRR male (SR). The results of G2 crosses to produce YYRR males and YYRR neofemales are shown in Table 5.9. In the control groups, male percentages of 100% were obtained, both of which were significantly different from 1:1 ($P<0.01$). No feminisation was achieved in the DES-treated groups. All red phenotypes were produced from these crosses as expected.

The consistency of sex ratios in progeny from crosses of three G2 YYRR males with three different normal XXrr females (SR) was investigated and the results are shown in Table 5.10. The proportion of males in progeny derived from the male of 00-13C-A8BS varied between 51.2-57.9% with a mean of 54.0%. These were not heterogeneous ($\chi^2_{(2)}=0.39$) and not significantly different from 1:1. On the other hand, the sex ratios of

Table 5.8. Sex ratios in control (C1-1C) and DES-treated (F1-1C) G1 progeny derived from a single pair mating of YYrr *O. niloticus* neofemale (Egypt-Swansea-Philippine) with an androgenetic YYRR male (Stirling red). In control, χ^2 values are for comparison against a 1:1 (F:M) sex ratio; in DES treatment, χ^2 values are for comparison to the sex ratio in the controls. (F: Female, M: Male, ***: P<0.001).

Families Tag no.	Codes	Number of F: M	% female	% male	$\chi^2_{(1)}$
Dam: 014 575 564 (YYrr)	C1-1C	0:27	0.0	100.0	27.00^{***}
Sire: 013 296 291 (YYRR)	F1-1C	78:28	44.8	55.21	45.06^{***}

Table 5.9. Sex ratios in control (C2-1C and C2-2C) and DES-treated (F2-1C and F2-2C) G2 progeny derived from two single pair matings between G1 YYRr *O. niloticus* neofemales and androgenetic YYRR males (Stirling red). In controls, χ^2 values are for comparison against a 1:1 (F: M) sex ratio; in DES treatments, χ^2 values are for comparisons to the sex ratio in the respective controls. (F: Female, M: Male, ***: P<0.001).

Families Tag no.	Codes	No. of F:M	% female	% male	$\chi^2_{(1)}$
Dam: 011 574 336 (YYRr)	C2-1C	0:21	0.0	100.0	21.00^{***}
Sire: 013 296 291 (YYRR)	F2-1C	0:37	0.0	100.0	-
Dam: 005 557 370 (YYRr)	C2-2C	0:18	0.0	100.0	18.00^{***}
Sire: 013 630 048 (YYRR)	F2-2C	0:66	0.0	100.0	-

Table 5.10. Sex ratios of progeny of three G2 YYRR males (from control group of C2-1C, see Table 5.9.) in breeding scheme 2 (see Fig. 5.9.). Males were crossed to three different normal XXrr females. Females were tested with a same normal XYrr male as control groups. Sex ratios of males were tested against a 1:1 ratio. (M: Male, F: female, a: Fisher exact test result, n.s.: not significant, *: P<0.05, **: P<0.01, ***: P<0.001)

Females (XXrr)	YYRR males			Control male (XYrr)
tag no.	00-013C-A8B8 (M:F) χ^2 (1:1)	00-013C-ABF7 (M:F) χ^2 (1:1)	00-013E-0CD2 (M:F) χ^2 (1:1)	015 302 791 (M:F) χ^2 (1:1)
00-013E-3362	(22:21) 0.02 n.s.	(37:0) 37.00^{***}	(32:0) 32.00^{***}	(6:8) 0.29 n.s.
00-013E-3245	(19:17) 0.11 n.s.	(35:0) 35.00^{***}	(28:0) 28.00^{***}	(14:11) 0.24 n.s.
00-013E-12E1	(22:16) 0.94 n.s.	(30:0) 30.00^{***}	(30:2) 24.50^{***}	17:19 0.11 n.s.
χ^2 heterogeneity	**0.39 n.s.**	-	**0.12 n.s.** [a]	**0.79 n.s.**

other two males were highly significantly different from a 1:1 sex ratio (P<0.001). The male percentages of offspring from 00-013C-ABF7 were 100% in all crosses with normal females whereas the male percentages of offspring from 00-013E-OCD2 varied between 93.8-100% with a mean of 97.9%. The χ^2 heterogeneity of the sex ratios produced by 015 302 791 control male was not significant ($\chi^2_{(2)}$=0.79).

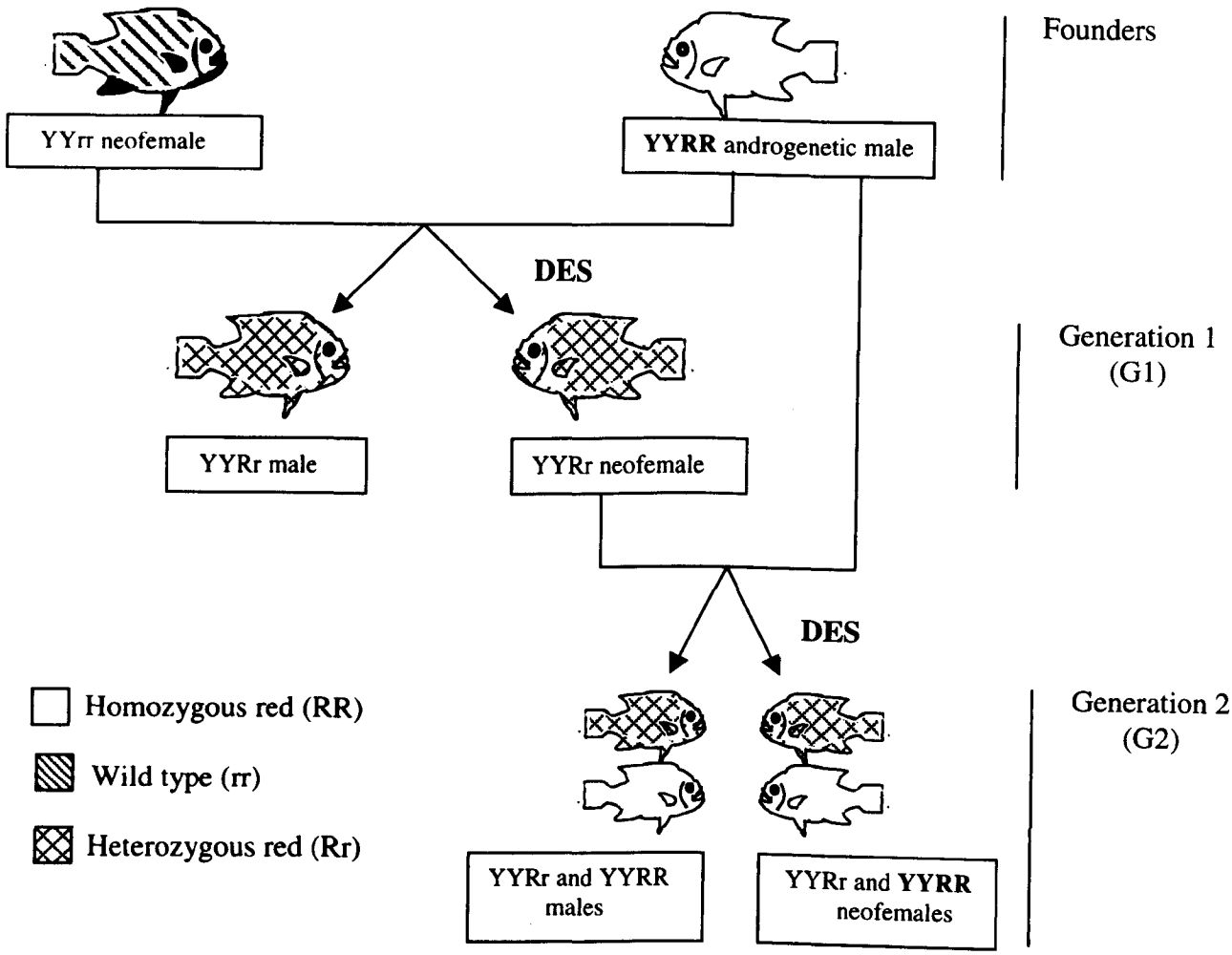


Figure 5.9. Schematic diagram of breeding scheme 2 for the production of YYRR males and YYRR neofemales by introgression of YYrr neofemales (Egypt-Swansea-Philippine) and androgenetic YYRR male (Stirling red) of *O. niloticus*.

No YYRR neofemales were produced using the breeding scheme 2 described above (2 ½ generations) since feminisation of progenies from G2 crosses derived from G1 YYRr x YYRR were unsuccessful. On the other hand, G1 crosses (from YYrr x YYRR crosses) and G2 crosses resulted in YYRr neofemales and YYRR males, respectively.

5.3.4. Production of YYRR males and YYRR neofemales by introgression of YYrr males (Egypt-Swansea-Philippine) and XXRR females (Stirling Red) in *O. niloticus*

The experimental design used to produce YYRR males and YYRR neofemales by introgression of YYrr males (ESP) and XXRR females (SR) in *O. niloticus* is schematically presented in Fig. 5.11. The sex ratios in control and DES-treated groups of G1 progeny derived from three single pair matings between XXRR females (SR) and YYrr males (ESP) are summarized in Table 5.11. In three DES-treated groups, the female proportions were 47.7-100 % with a mean of 82.6 %. The calculated values of contingency χ^2 showed that proportions of females in all DES-treated groups were significantly different from their respective controls ($P<0.001$). Male percentages of 100% were obtained in all control crosses were significantly different from 1:1 sex ratio ($P<0.001$). The phenotype of fish was all red as expected from these crosses.

The proportions of males and females in control and DES-treated G2 progenies derived from five single pair matings between G1 XYRr neofemales with normal XYRR males (SR) are shown in Table 5.12. The sex ratios were significantly different from the expected sex ratio of 1:3 (F:M) in the control groups C2-1A ($P<0.05$) and C2-3A ($P<0.001$). However, the sex ratios in the control groups C2-2A, C2-4A and C2-5A were not significantly different from the expected sex ratio of 1:3 ($P>0.05$).

The proportions of females in the DES-treated group of F2-1A and F2-2A were highly significantly different from their respective groups ($P<0.001$) whereas, the DES-treated groups of F2-3A, F2-4A and F2-5A did not differ significantly from their control groups in terms of female percentages ($P>0.05$). However, fish numbers in the control

Table 5.11. Sex ratios in control (C1-1A, C1-2A and C1-3A) and DES-treated (F1-1A, F1-2A and F1-3A) G1 progeny derived from three single pair matings of normal XXRR *O. niloticus* females (Stirling red) with YYrr males (Egypt-Swansea-Philippines). In controls, χ^2 values are for comparison against a 1:1 (F: M) sex ratio; in DES treatments χ^2 values are for comparisons to the sex ratio in the respective controls. (F: Female, M: Male, ***: P<0.001)

Families Tag no.	Codes	Number of F: M	% female	% male	$\chi^2_{(1)}$
Dam: 000 621 331 (XXRR) Sire: 013 313 104 (YYrr)	C1-1A	0:72	0.0	100.0	72.00***
	F1-1A	21:23	47.7	52.3	38.80***
Dam: 014 303 075 (XXRR) Sire: 014 571 512 (YYrr)	C1-2A	0: 29	0.0	100.0	29.00***
	F1-2A	49:0	100.0	0.0	73.78***
Dam: 014 106 788 (XXRR) Sire: 013 891 091 (YYrr)	C1-3A	0: 43	0.0	100.0	43.00***
	F1-3A	13:0	100.0	0.0	50.53***

Table 5.12. Sex ratios in control (C2-1A, C2-2A, C2-3A, C2-4A and C2-5A) and DES-treated (F2-1A, F2-2A, F2-3A, F2-4A and F2-5A) G2 progeny derived from five single pair matings between G1 XYRr *O. niloticus* neofemales and normal XYRR males (Stirling red). In controls, χ^2 values are for comparison against expected 1:3 (F: M) sex ratio; in DES treatments χ^2 values are for comparisons to the sex ratio in the respective controls. (F: Female, M: Male, a: Original family of dam (see Table 5.11.), b: Fisher Exact test result since the expected number was less than 5, *: P<0.05, **: P<0.01, ***: P<0.001, n.s.: not significant).

Families Tag no.	Codes	Number of F:M	% female	% male	$\chi^2_{(1)}$
Dam: 011 105 002 (XYRr) (F1-2A)[a] Sire: 014 300 264 (XYRR)	C2-1A	6:42	16.0	84.0	4.00*
	F2-1A	46:4	92.0	8.0	58.99***
Dam: 002 046 351 (XYRr) (F1-2A)[a] Sire: 015 296 120 (XYRR)	C2-2A	7:43	14.0	86.0	3.23 n.s.
	F2-2A	33:13	71.7	28.3	30.53***
Dam: 011 587 004 (XYRr) (F1-2A)[a] Sire: 014 300 261 (XYRR)	C2-3A	27:31	46.5	53.5	14.37***
	F2-3A	27:14	65.9	34.1	2.87 n.s
Dam: 001 094 829 (XYRr) (F1-3A)[a] Sire: 013 110 524 (XYRR)	C2-4A	2:6[b]	25.0	75.0	0.43 n.s.
	F2-4A	5:5	50.0	50.0	0.22 n.s
Dam: 002 374 516 (XYRr) (F1-3A)[a] Sire: 013 555 839 (XYRR)	C2-5A	0:3[b]	0.0	100.0	0.50 n.s.
	F2-5A	32:1	96.9	3.1	0.00 n.s

groups of C2-4A and C2-5A was very low for a good comparison to the DES-treated groups of F2-4A and F2-5A, respectively. The all red phenotype was obtained in all crosses as expected.

No YY males from G2 control crosses were obtained from the fourteen males tested (Table 5.13.) which is significantly different from the 50% of YYRR genotypes expected from these crosses (based on testing of only RR males, see Table 5.15.). One out of these fourteen control males gave all female progenies designated as XXRR neomale.

A total of fifteen DES-treated homozygous red females were progeny tested from G2 crosses and only one was identified as a YYRR neofemale (Table 5.14.). This was significantly different from the expected 33.3% YYRR genotypes in these crosses ($P<0.05$) (Based on only RR neofemales, see Table 5.15.). Eight of the fifteen DES-treated females were identified as XYRR neofemales (Table 5.14.) which was not significantly different from the expected 33.3% XY genotypes ($P>0.05$, see Table 5.15.). Six of the fifteen DES-treated females were designated as XXRR (Table 5.14.) which was not significantly different from the expected 33.3% XXRR genotype from these crosses (see Table 5.15.). The result of progeny testing of control males and DES-treated females are summarized in Table 5.15. (Based on testing of only RR fish).

Fig 5.10. shows the frequency distribution of sex ratios in progeny testing of G2 YYRR *O. niloticus* males (A) and G2 YYRR neofemales (B) from control and DES-treated G2 crosses, respectively, (between G1 XYRr neofemale with normal XYRR male crosses).

Table 5.13. Results of progeny testing to identify YYRR *O. niloticus* males from G2 control crosses (from G1 XYRr neofemale x normal XYRR male crosses, see Fig. 5.11.). Progeny testing was based on only RR males. (F: Female, M: Male, a:χ^2 contingency test since the sex ratio of the respective control was significantly different from a 1:1 ratio *: P<0.05, **: P<0.01, ***: P<0.001, for original family of fish see Table 5.12.)

XY/YY RR males tag no. (original family)	No. of fry sexed	No. of M:F	% male	χ^2 (1) (1:1)	Predicted genotypes	Sex ratio of respective control M:F
00-013E-14CD (C2-2A)	6	4:2	66.7	0.66	XY	13:17
00-0136-05A2 (C2-2A)	40	0:40	0.0	40.00^{***}	XX	13:17
00-013E-3365 (C2-2A)	14	6:8	42.9	1.25^{a}	XY	$9:31^{***}$
00-013E-3FF5 (C2-2A)	40	26:12	65.0	5.16^{*}	XY	1:5
00-013C-8835 (C2-2A)	13	6:7	46.2	0.08	XY	16:14
00-013E-4344 (C2-3A)	4	2:2	50.0	0.00	XY	22:14
00-013C-B3A1 (C2-3A)	29	20:9	69.0	4.17^{*}	XY	22:14
00-013E-48E3 (C2-3A)	50	34:16	68.0	6.48^{*}	XY	22:14
00-013E-3BB8 (C2-3A)	36	21:15	58.3	1.00	XY	22:14
00-013D-F544 (C2-3A)	38	16:22	42.1	0.95	XY	13:17
00-013E-4344 (C2-3A)	40	13:27	32.5	4.9^{*}	XY	13:17
00-013E-58C8 (C2-4A)	14	8:6	57.1	0.29	XY	22:14
00-0136-0272 (C2-4A)	36	17:19	47.2	0.11	XY	22:14
00-013E-083B (C2-4A)	42	22:20	52.4	0.4	XY	17:19

Table 5.14. Results of progeny testing to identify YYRR *O. niloticus* neofemales from DES-treated G2 progeny (from G1 XYRr neofemale x normal XYRR male crosses, see Fig. 5.11.). Progeny testing based on only RR neofemales. (F: Female, M: Male, *: P<0.05, **: P<0.01, ***: P<0.001, for original family of fish see Table 5.12.)

XY/YY RR females tag no. (Original family)	No. of fry sexed	No. of M:F	% male	χ^2 (1) (1:1)	Predicted genotypes
00-013C-A34F (F2-1A)	36	0:36	0.0	36.00***	XX
00-013E-3F1E (F2-1A)	14	10:4	71.4	2.57	XY
00-0136-0868 (F2-2A)	32	11:21	34.4	3.13	XY
00-013E-3706 (F2-2A)	25	0:25	0.0	25.00***	XX
00-013C-AB38 (F2-3A)	50	43:7	86.0	25.92***	YY
00-013E-09B5 (F2-3A)	32	16:16	50.0	0.00	XY
00-013D-EECD (F2-3A)	36	1:35	2.8	32.11***	XX
00-013E-3EE8 (F2-3A)	38	24:14	63.2	3.41	XY
00-013E-0F53 (F2-3A)	39	17:22	43.6	0.64	XY
00-013D-FA3C (F2-3A)	38	20:18	52.6	0.11	XY
00-0137-9D78 (F2-3A)	30	1:29	3.3	26.13***	XX
00-0F7-76ED (F2-3A)	38	23:15	60.5	1.68	XY
00-013D-F53A (F2-3A)	32	3:29	9.4	21.13***	XX
00-013C-B00D (F2-4A)	24	0:24	0.0	24.00***	XX
00-013D-ED8F (F2-4A)	34	12:22	35.3	2.94	XY

Table 5.15. Summary of results of progeny testing to identify G2 YYRR *O. niloticus* males and YYRR neofemales from control and DES-treated G2 progeny, respectively, (from XYRr neofemale x normal XYRR male crosses, see Fig.5.11.). Progeny testing was based on only RR males and RR females.

Description	**Males**	**Females**
No. of families from which fish tested	4	4
No. of fish tested	14	15
No. of fry sexed	402	498
Average family size	29	33.2
No. of genotypes classified as XXRR	1	6
No. of genotypes classified as XYRR	13	8
No. of genotypes classified as YYRR	0	1
Expected no. of YYRR genotypes	7 (50%)	5 (33.3%)
χ^2 for observed versus expected no. of YYRR	14.00^{***}	5.97^{*}
No. of fish not classified	0	0
Male percentage in progeny from XYRR genotype	32.5-66.7	34.4-71.4
Mean male percentage in progeny from XYRR genotype	53.6	51.4
Male percentage in progeny from YYRR genotype	-	86.0
Mean male percentage in progeny from YYRR genotype	-	-

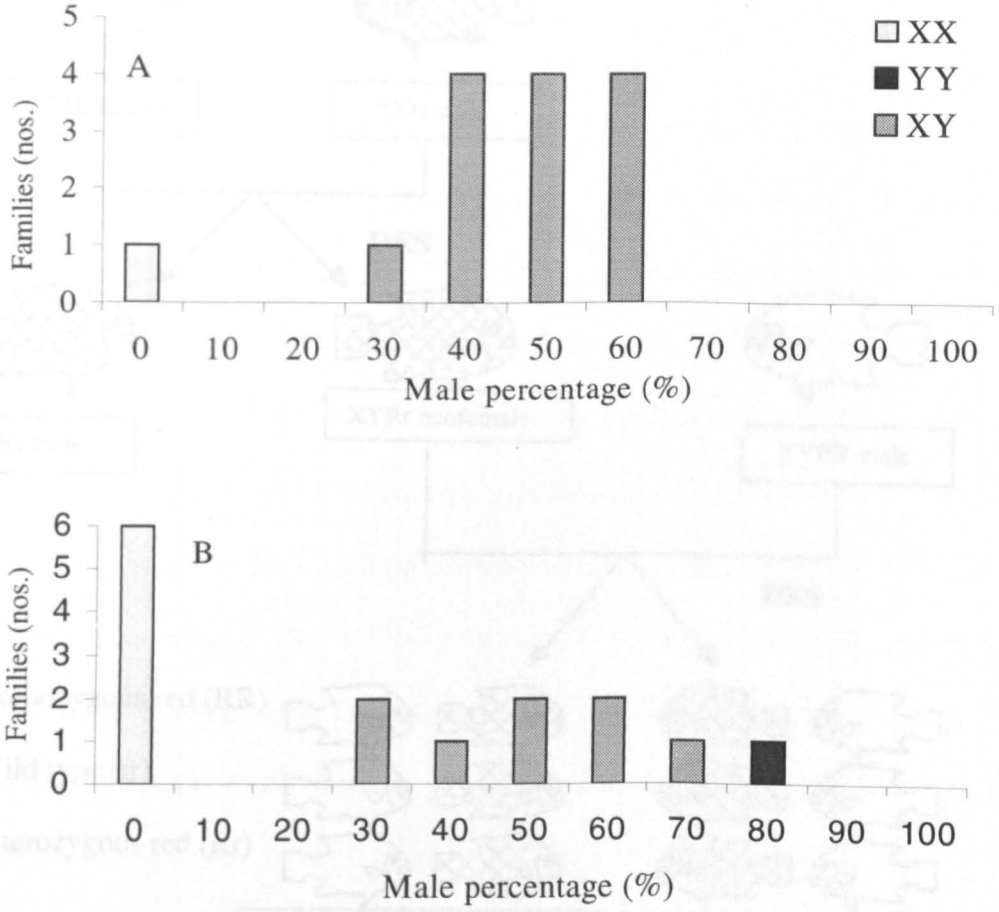


Figure 5. 10. Frequency distribution of sex ratios in progeny testing of G2 YYRR *O. niloticus* males (A) and G2 YYRR neofemales (B) from control and DES-treated G2 crosses, respectively, (between G1 XYRr neofemale with normal XYRR male crosses). All males and neofemales were crossed to normal XXrr females and an XXrr neomale, respectively.

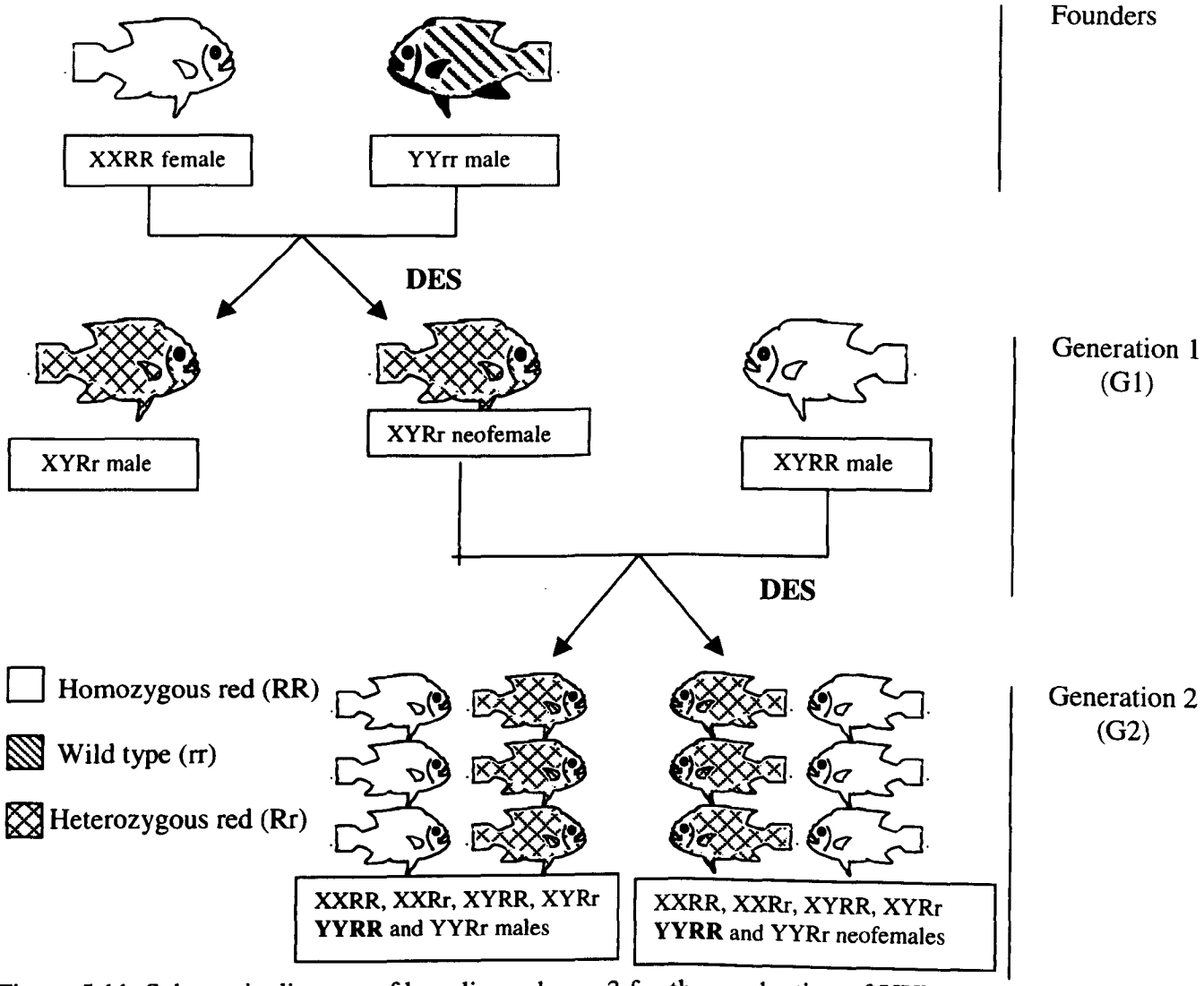


Figure 5.11. Schematic diagram of breeding scheme 3 for the production of YYRR males and YYRR neofemales by introgression of YYrr males (Egypt-Swansea-Philippine) and normal XXRR females (Stirling red) of *O. niloticus*.

No YYRR males were produced while only one YYRR neofemales was obtained in this breeding scheme (2 ½ generations) which aimed to integrate the YY genotype with RR genotype in order to produce YYRR males and YYRR neofemales. The feminisation of progenies from G1 crosses between XXRR x YYrr and from G2 crosses between XYRr x XYRR were successful.

5.3.5. Production of YYRR males and YYRR neofemales by introgression of YYrr neofemales (Egypt-Swansea-Philippine) and XYRR males (Stirling Red) in *O. niloticus*

The experimental design used to produce YYRR males and YYRR neofemales by introgression of YYrr neofemales (ESP) and XYRR males (SR) in *O. niloticus* is schematically presented in Fig. 5.14. (page 255). The sex ratios in control and DES-treated groups of G1 progenies derived from four single pair matings between YYrr neofemales (ESP) and XYRR males (SR) are depicted in Table 5.16. In all control groups, sex ratios were 100% male and significantly different from 1:1 sex ratio ($P<0.001$), as expected. The female proportion in DES-treated groups ranged between 56.8-91.6% with a mean of 79.1%. The proportion of females in all DES-treated groups was significantly different from the respective controls (at $P<0.001$ in the group of F1-1B, F1-3B and F1-4B and at $P<0.01$ in the group of F1-2B). The expected red phenotype was obtained in all crosses.

Of twelve control males tested from G1 crosses, four males were identified as YYRr males (Table 5.17.), which was not significantly different from the 50% expected YYRr genotypes in these crosses ($P>0.05$, see Table 5.19.). Percentages of males in progeny from individuals classified as YYRr males significantly differed from 1:1 ratio ($P<0.001$) ranged between 76.9-84.6% with a mean of 80.9%. Seven remaining males were designated as normal XYRr males since these males produced progeny with proportions of males ranging from 32.3-73.1% with a mean of 62.1%, not significantly different from a 1:1 ratio.

Table 5.16. Sex ratios in controls (C1-1B, C1-2B, C1-3B and C1-4B) and DES-treated (F1-1B, F1-2B, F1-3B and F1-4B) G1 progeny derived from four single pair matings of YYrr *O. niloticus* neofemales (Egypt-Swansea-Philippines) with normal XYRR males (Stirling red). In controls, $\chi 2$ values are for comparison against a 1:1 (F:M) sex ratio; in DES treatments $\chi 2$ values are for comparisons to the sex ratio in the respective controls. (F: Female, M: Male, *: P<0.05, **: P<0.01, ***: P<0.001)

Families Tag no.	Codes	Number of F: M	% female	% male	$\chi^2_{(1)}$
Dam: 012 618 313 (YYrr) Sire: 013 555 839 (XYRR)	C1-1B	0:47	0.0	100.0	47.00^{***}
	F1-1B	11:5	91.6	8.4	34.52^{***}
Dam: 014 312 864 (YYrr) Sire: 013 110 524 (XYRR)	C1-2B	0: 11	0.0	100.0	11.00^{***}
	F1-2B	46:35	56.8	43.2	10.32^{**}
Dam: 014 575 564 (YYrr) Sire: 014 300 261 (XYRR)	C1-3B	0: 28	0.0	100.0	28.00^{***}
	F1-3B	63:12	84.0	16.0	57.08^{***}
Dam: 007 336 335 (YYrr) Sire: 014 300 261 (XYRR)	C1-4B	0: 24	0.0	100.0	24.00^{***}
	F1-4B	17:5	84.0	16.0	26.19^{***}

Table 5.17. Results of progeny testing to identify G1 YYRr *O. niloticus* males from control groups of G1 crosses (between YYrr neofemales x normal XYRR males, see Fig. 5.14.). YY males are denoted as those producing a sex ratio significantly different from a 1:1 ratio at P<0.001. (*: P<0.05, **: P<0.01, ***: P<0.001, for original family of fish (see Table 5.16.)

XY/YY Rr males tag no. (Original family)	No. of fry sexed	No. of M:F	% male	χ^2 (1) (1:1)	Predicted genotypes
007 009 888 (C1-1B)	21	10:11	32.3	0.04	XY
000 880 579 (C1-1B)	27	19:9	70.3	3.74	XY
000 290 054 (C1-1B)	30	21:9	70.0	4.80*	XY
000 261 083 (C1-1B)	19	10:9	52.6	0.05	XY
010 114 791 (C1-2B)	26	19:7	73.1	5.53*	XY
001 030 326 (C1-2B)	52	42:10	80.8	19.70***	YY
000 520 805 (C1-2B)	52	44:8	84.6	25.00***	YY
000 807 096 (C1-2B)	35	25:10	71.4	6.40*	XY
000 885 523 (C1-2B)	52	40:12	76.9	15.07***	YY
005 610 566 (C1-3B)	32	26:6	81.3	12.50***	YY
014 319 328 (C1-3B)	20	13:7	65.0	1.80	XY
010 317 095 (C1-3B)	32	25:7	78.1	10.12**	??

Two of the nineteen DES-treated females from G1 crosses were identified as YYRr neofemales (Table 5.18) which was significantly different from the 50% expected YYRr genotype in these crosses (see Table 5.19.). The male percentages of offspring from two YYRr neofemales were 74.5% and 76.8%. Significantly higher numbers XYRr neofemales were produced (n=16) than the expected number of 9.5 XYRr neofemales in these crosses ($P<0.01$) (see Table 5.19.). The male percentages of offspring from XYRr neofemales ranged between 34.0-77.7% with a mean of 75.65%. Progeny testing results of untreated males and neofemales from G1 crosses derived from between YYrr neofemales (ESP) with normal XYRR males (SR) are summarised in Table 5.19.

Fig 5.12. shows the frequency distribution of sex ratios in progeny testing of G1 YYRr *O. niloticus* males (A) and G1 YYRr neofemales (B) from control and DES-treated G1 progeny, respectively, (between YYrr neofemale x normal XYRR male crosses). No clear distribution was observed for male percentages of G1 YYRr males (A), probably resulted from low number of fish used with sample.

Two identified YYRr neofemale (013 284 559) and YYRr male (005 610 565) originating from the G1 progeny were crossed to generate G2 cross and only red fish were used for feminisation. The sex ratios in the control and DES-treatment of G2 cross are presented in Table 5.20. The male percentage in the control cross was 97.2% and this sex ratio was significantly different from a 1:1 ratio ($P<0.001$). A female percentage of 51.0% was obtained in the DES-treated group. The χ^2 contingency value calculated for comparison of the DES-treated group with the control was highly significant ($P<0.001$), indicating a significant level of feminisation. Since only red fish were used for feminisation, only the colour ratio of control group was given in Table 5.20. The colour

Table 5.18. Results of progeny testing to identify G1 YYRr *O. niloticus* neofemales from DES-treated G1 progeny (between YYrr neofemale x normal XYRR male crosses, see Fig. 5.14). YY females are denoted as those producing sex ratio significantly different from expected 1:1 ratio at P<0.001. (*: P<0.05, **: P<0.01, ***: P<0.001, for original family of fish see Table 5.16.)

XY/YY Rr females tag no. (Original family)	No. of fry sexed	No. of M:F	% male	χ^2 (1) (1:1)	Predicted genotypes
013 284 559 (F1-1B)	56	43:13	76.8	16.06***	YY
013 317 300 (F1-1B)	31	15:11	48.4	0.62	XY
014 572 600 (F1-1B)	25	11:14	44.0	0.36	XY
013 107 104 (F1-1B)	17	5:12	47.0	1.44	XY
009 592 335 (F1-1B)	69	44:25	63.8	5.23**	??
011 797 381 (F1-2B)	20	8:12	40.0	0.62	XY
008 565 364 (F1-2B)	55	41:14	74.5	13.25***	YY
000 000 001 (F1-2B)	48	23:25	48.0	0.08	XY
013 278 044 (F1-2B)	9	7:2	77.7	2.76	XY
009 283 829 (F1-3B)	11	4:7	36.0	0.81	XY
010 054 015 (F1-3B)	52	34:18	65.4	4.92*	XY
001 258 549 (F1-4B)	26	12:14	46.2	0.15	XY
000 616 814 (F1-4B)	31	20:11	64.5	1.31	XY
014 296 107(F1-4B)	44	15:29	34.0	4.44*	XY
013 622 301 (F1-4B)	52	36:16	69.2	3.85*	XY
011 559 059 (F1-4B)	22	13:9	59.0	0.72	XY
005 100 858 (F1-4B)	23	9:14	39.0	1.08	XY
000 000 002 (F1-4B)[a]	43	23:20	53.5	0.2	XY
012 554 075 (F1-4B)	28	15:13	53.6	0.15	XY

Table 5.19. Summary of results of progeny testing to identify G1 YYRr *O. niloticus* males and G1 YYRr neofemales from control and DES-treated G1 progeny, respectively, (from YYrr neofemale x normal XYRR male, crosses, see Fig. 5.14.)

Description	**Males**	**Females**
No. of families from which fish tested	3	4
No. of fish tested	12	19
No. of fry sexed	398	662
Average family size	33.2	34.8
No. of genotypes classified as XYRr	7	16
No. of genotypes classified as YYRr	4	2
Expected no. of YYRR genotypes (50%)	6	9.5
χ^2 for observed versus expected no. of YYRr	1.33	11.8^{***}
No. of fish not classified	1	1
Male percentage in progeny from XYRr genotype	32.3-73.1	34.0-77.7
Mean male percentage in progeny from XYRr genotype	62.1	51.6
Male percentage in progeny from YYRr genotype	76.9-84.6	74.5-76.8
Mean male percentage in progeny from YYRr genotype	80.9	75.7

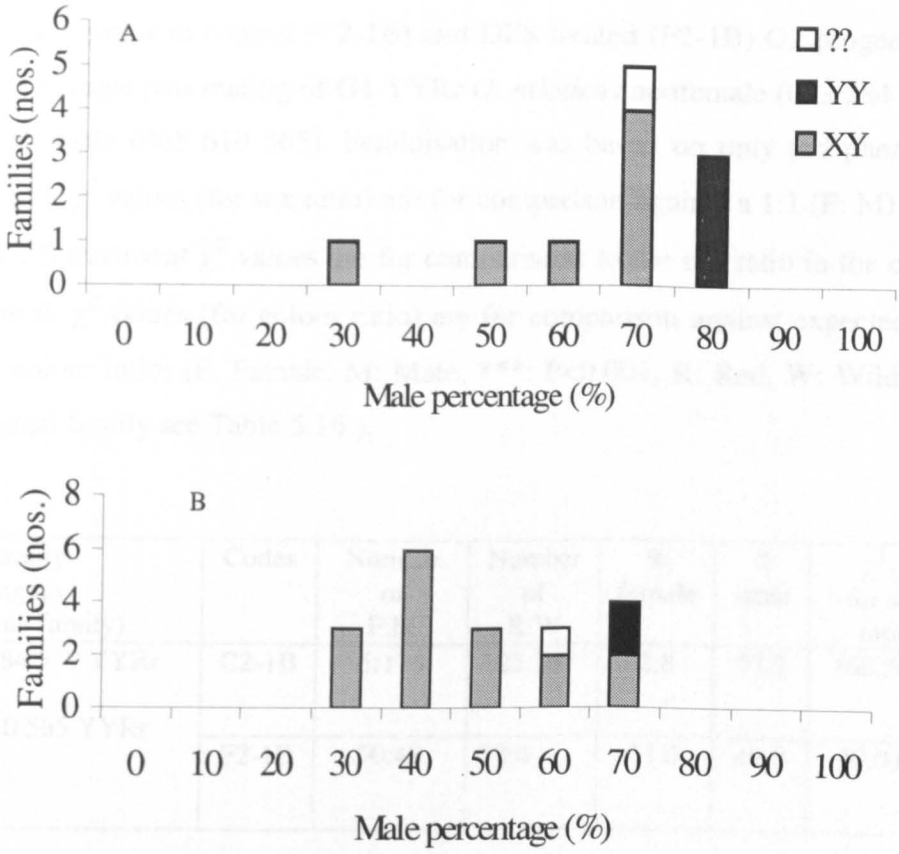


Figure 5.12. Frequency distribution of sex ratios in progeny testing of G1 YYRr *O. niloticus* males (A) and YYRr neofemales (B) from control and DES-treated G1 progeny, respectively, (from YYrr neofemale x normal XYRR male crosses). All males and females were crossed to normal XXrr females and an XXrr neomale, respectively.

Table 5.20. Sex ratios in control (C2-1B) and DES treated (F2-1B) G2 progeny derived from a single pair mating of G1 YYRr *O. niloticus* neofemale (013 284 559) with YYRr male (005 610 565). Feminisation was based on only red phenotype. In control, χ^2 values (for sex ratio) are for comparison against a 1:1 (F: M) sex ratio; in DES treatment χ^2 values are for comparisons to the sex ratio in the control. In control, χ^2 values (for colour ratio) are for comparison against expected 3:1 (R: W) colour ratio; (F: Female, M: Male, ***: P<0.001, R: Red, W: Wild type, for original family see Table 5.16.).

Family Tag no. (Original family)	Codes	Number of F:M	Number of R:W	% female	% male	$\chi^2_{(1)}$ for sex ratio	$\chi^2_{(1)}$ for colour ratio
Dam: 013 284 559 YYRr (F1-1B) Sire: 005 610 565 YYRr (F1-3B)	C2-1B	5:175	125:55	2.8	97.2	160.56***	2.96 n.s.
	F2-1B	50:48	98:0	51.0	49.0	90.04***	-

ratio in control group was not significantly different from the expected 3:1 (Red: Wild type).

Of seven control males from G2 cross, five males were designated as YYRR males (Table 5.21.). Although this was not significantly different from the expected 100% YYRR genotype, considering that all YYRR genotype was expected, the number (five YYRR) was much lower than expected seven YYRR genotype in this crosses (see Table 5.23). Male percentages in progeny from these YYRR males were ranged between 77.5-100 % with a mean of 95.5%.

A total of five DES sex reversed females from G2 cross were tested and three of them were classified as YYRR neofemales (Table 5.22.) which was not significantly different but much lower than the expected 100% YYRR genotype in this cross (P<0.05,

Table 5.21. Results of progeny testing to identify G2 YYRR *O. niloticus* males from G2 control cross (between G1 YYRr neofemale (013 284 559) with G1 YYRr male (005 610 565) cross, see Fig. 5.14.). Progeny testing was based on only RR males. YY males are denoted as those producing sex ratios significantly different from 1:1 ratio at P<0.001. (M; male, F; female, *: P<0.05, **: P<0.01, ***: P<0.001, for original family of fish see Table 5.20.)

XY/YY RR males tag no. (Original family)	No. of fry sexed	No. of M:F	% male	χ^2 (1) (1:1)	Predicted genotypes	Sex ratio of respective control M:F
00-012C-12DD (C2-1B)	32	32:0	100.0	32.00***	YY	22:16
00-013E-1045 (C2-1B)	40	31:9	77.5	12.10***	YY	22:16
00-013E-0DAY (C2-1B)	30	30:0	100.0	30.00***	YY	22:16
00-013C-AD8C (C2-1B)	36	19:17	52.7	0.11	??	22:16
00-013E-34AB (C2-1B)	34	34:0	100.0	34.00***	YY	9:31***
00-013E-3246 (C2-1B)	30	30:0	100.0	30.00***	YY	22:16
00-012C-0EEC (C1-1B)	44	20:24	45.5	0.36	??	22:16

Table 5.22 . Results of progeny testing to identify G2 YYRR *O. niloticus* neofemales from DES-treated G2 progeny (between G1 YYRr neofemale (013 284 559) x G1 YYRr male (005 610 565) cross, see Fig. 5.14.). Progeny testing was based on only RR neofemales. YY females were denoted as those producing sex ratios significantly different from expected 1:1 ratio at P<0.001. (M; male, F; female, *: P<0.05, **: P<0.01, ***: P<0.001, for original family of fish see Table 5.20.).

XY/YY RR females tag no. (Original family)	No. of fry sexed	No. of M:F	% male	χ^2 (1) (1:1)	Predicted genotypes
00-013E-0F66 (F2-1B)	24	24:0	100.0	24.00***	YY
00-013E-DD73 (F2-1B)	34	11:23	32.4	4.24*	??
00-013E-49D2 (F2-1B)	32	32:0	100.0	32.00***	YY
00-012F-39D7 (F2-1B)	15	11:4	73.3	3.26	??
00-013E-0F66 (F2-1B)	30	30:0	100.0	30.00***	YY

5.23.). The male percentage of progeny of YYRR neofemales was 100%. Progeny testing results of control G2 YYRR males and YYRR neofemales are summarised in Table 5.23. (Based on testing of only RR fish).

Fig 5.13. shows the frequency distribution of sex ratios in progeny testing of G2 YYRR *O. niloticus* males (A) and G2 YYRR neofemales from control and DES-treated G2 progeny, respectively, (from G1 YYRr neofemale x G1 YYRr male cross).

Table 5.23. Summary results of progeny testing to identify G2 YYRR *O. niloticus* males and YYRR neofemales from control and DES-treated G2 progeny, respectively, (from G1 YYRr neofemale (013 284 559 YYRr) x YYRr male (005 610 565) cross, see Fig. 5.14.). Progeny testing was based on only RR males and RR neofemales.

Description	**Males**	**Females**
No. of families from which fish tested	1	1
No. of fish tested	7	5
No. of fry sexed	246	135
Average family size	35.1	27
No. of genotypes classified as YYRR	5	3
Expected no. of YYRR genotypes (100%)	7	5
Exact probabilty for binomial test	0.2	0.2
No. of fish not classified	2	2
Male percentage in progeny from YYRR genotype	77.5-100.0	100.0
Mean male percentage in progeny from YYRR genotype	95.5	100.0

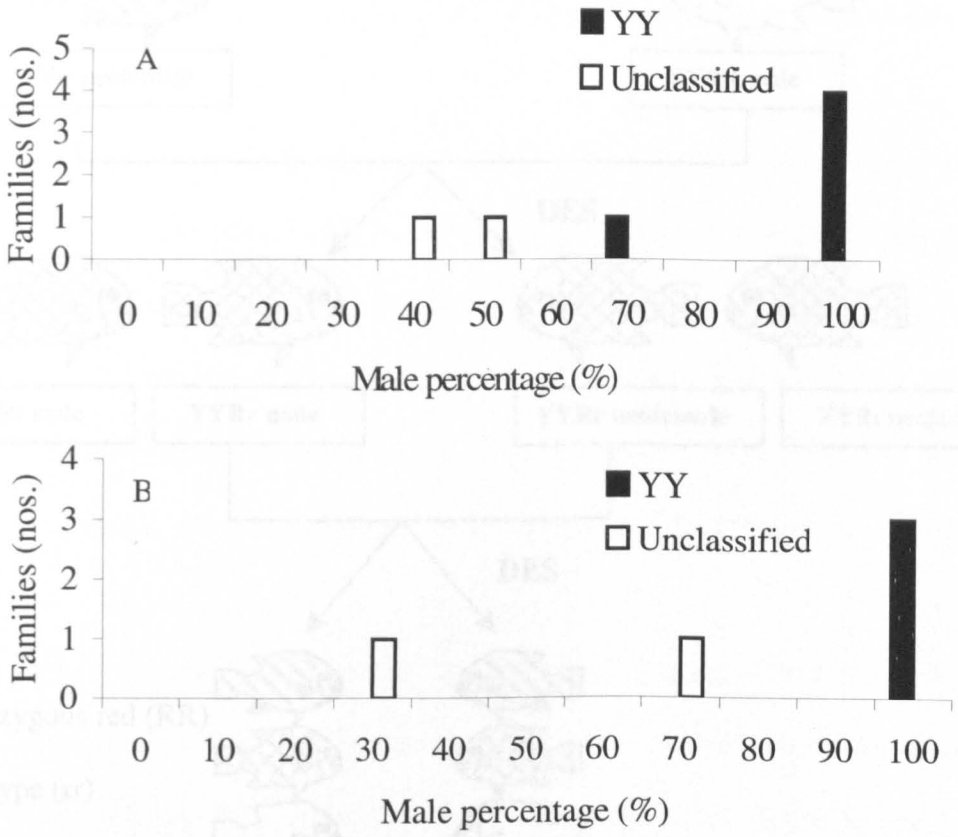


Figure 5.13. Frequency distribution of sex ratios in progeny testing of G2 YYRR *O. niloticus* males (A) and G2 YYRR neofemales (B) from control and DES-treated G2 progeny, respectively, (from G1 YYRr neofemale x G1 YYRr male cross).All males and neofemales were crossed to normal XXrr females and an XXrr neomale, respectively.

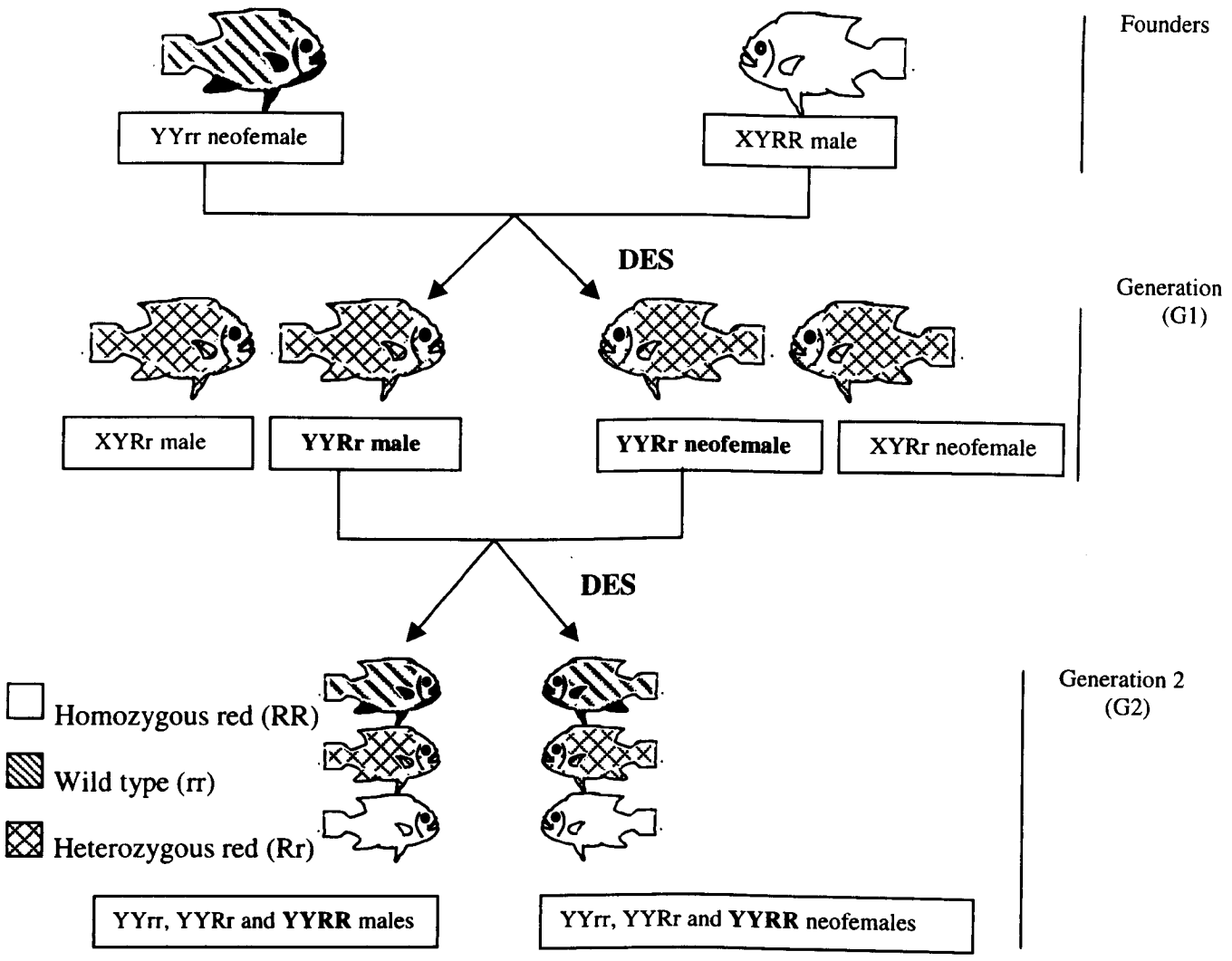


Figure 5.14. Schematic diagram of breeding scheme 4 for the production of YYRR males and YYRR neofemales by introgression of YYrr neofemales (Egypt-Swansea-Philippine) and normal XYRR male (Stirling red) of *O. niloticus*.

Although the breeding scheme described above took longer (3 generations) compared to other breeding schemes in order to combine YY genotype and RR genotype in a single strain, the number and male percentages of YYRR males and YYRR neo females were higher. A total of 5 YYRR males and 3 YYRR neofemales with an average male percentages of 95.5% and 100.0% were produced, respectively. YY genotype were successfully feminised.

5.3.6. Summary of sex ratios produced by XY and YY genotypes in male and female of *O. niloticus*

The male percentages in the progeny testing of XY and YY genotypes in male and female *O. niloticus* were pooled and the results are presented as a frequency distribution in Fig. 5.11. The frequency distribution of family sex ratio from XY males and XY neofemales was normally distributed. On the other hand, the sex ratio frequency distribution for the progeny testing of YY males was bimodal with two peaks at 80.0% and 100%. The family sex ratio frequency distribution of YY neofemales showed a single mode.

The progeny testing of XY males with normal XX females produced sex ratios slightly skewed to male. The male percentage from all twenty XY males varied between 32.5-73.1% with a mean of 56.6±2.62%, significantly greater than the predicted 1:1 ratio ($\chi^2_{(1)}$=13.40; P<0.001). Overall progeny testing of XY neofemales produced a mean male percentage of 50.9±2.29% which was not significantly different from the expected 1:1 ratio ($\chi^2_{(1)}$=0.17).

Progeny testing of YY males and YY neofemales, excluding those males and females from YY x YY crosses, produced sex ratios ranging from 76.9-84.6% with a mean of 80.9% and 74.5-86.9% with a mean of 80.0%, respectively. YY males and YY neofemales from the YY x YY crosses gave a higher mean male percentage of 96.4% and 100%, respectively. Mean male percentages of YY males and YY neofemales from all crosses were 91.2±2.80% and 88.2±3.76%, respectively.

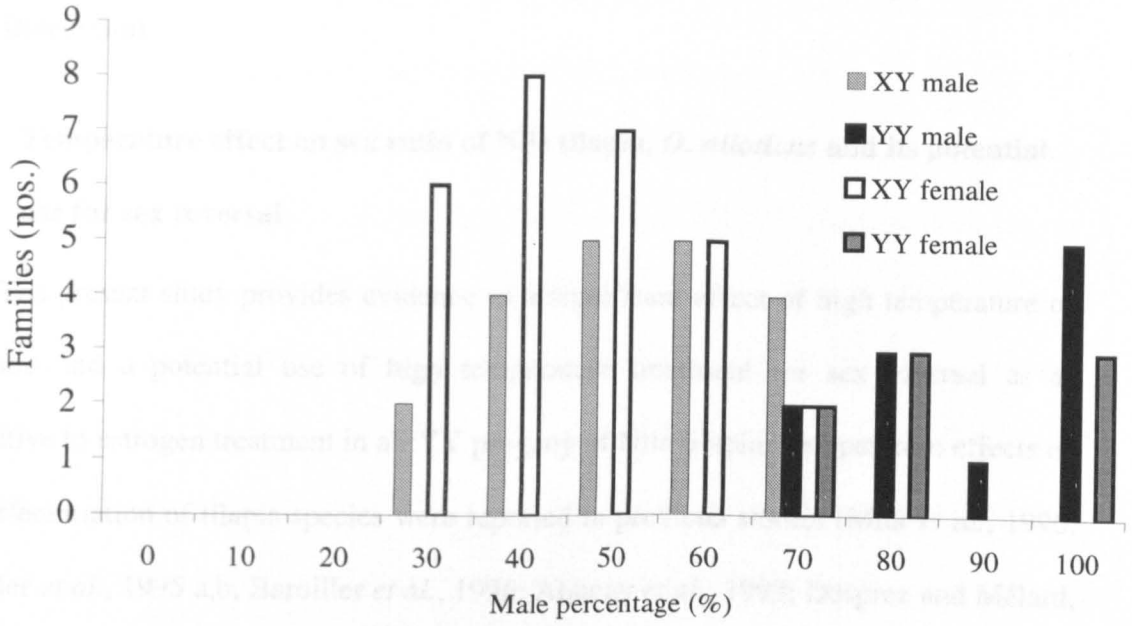


Figure 5.15. Frequency distribution of pooled male percentages in the progeny testing for XY and YY genotypes in male and female *O. niloticus*. Males and females were crossed to normal XX females and an XX neomale, respectively.

5.4. Discussion

5.4.1. Temperature effect on sex ratio of Nile tilapia, *O. niloticus* and its potential use for sex reversal

The present study provides evidence of a significant effect of high temperature on sex ratio and a potential use of high temperature treatment for sex reversal as an alternative to estrogen treatment in all YY progeny of Nile tilapia. Temperature effects on sex differentiation of tilapia species were reported in previous studies (Mair *et al.*, 1990; Baroiller *et al.*, 1995 a,b; Baroiller *et al.*, 1996; Abucay *et al.*, 1999; Desprez and Mélard, 1998). Sex reversal effects of high temperature treatment were also demonstrated on putative all-females (Baroiller *et al.*, 1996; Abucay, 1999) and YY males (Abucay *et al.*, 1999) progeny of *O. niloticus* in the direction of male and female, respectively.

In the present study, a significantly lower mean survival rate of 62.9±9.82% was observed in the heat treated groups compared to the survival rate of 97.0±0.85% in the controls and 97.3±0.82% in the DES treatment groups. These results suggest that YY male progeny of *O. niloticus* are more susceptible to high temperature. A similar observation on the sensitivity of YY males to high temperature treatment was reported by Abucay *et al.* (1999) in same strain of *O. niloticus*. In their work, a significantly lower mean survival rate of 53.0% was obtained in heat treated group (36°C) compared with mean survival rate of 90.67% in the control.

No female progeny were observed in any control group in crosses between YY x YY genotypes. This is predicted from the sex-determination mechanism, of a predominantly monofactorial genotypic system with male heterogamety and female

homogamety suggested by several authors (Penman *et al.*, 1987; Shah, 1988; Mair *et al.*, 1991a; Muller-Belecke and Horstgen-Schwark, 1995; Mair *et al.*, 1997 and Chapter 2 in this thesis). On the other hand, a mean female percentage of 32.0±5.21% produced in heat treated groups of all YY male progeny indicated that high temperature does change the sex of some YY male *O. niloticus* in the direction to female. Mean female percentages of 32.0±5.21% in this study were comparable to the mean female percentages of 49.22% (ranging from 0-94.44%) obtained by Abucay *et al.* (1999) in heat treated all YY male progenies from the same strain (ESP) of *O. niloticus* derived from thirteen families. On the other hand, in the same study, lower female percentages ranging from 0-11.54% with a mean of 1.42% were observed in progenies subjected to heat treatment at 36°C from the crosses of YY neofemales from ESP with YY males from Egypt-ICLARM strains of *O. niloticus*. The authors attributed the different sensitivity of pure-bred (ESP x ESP) and crossbred (ESP x Egypt-ICLARM) YY males reared at high temperature to the level of inbreeding of the Egypt-Swansea strain which is affecting their fitness and developmental stability and making them more sensitive to environmental extremes.

A mean female percentage of 33.8±1.5% was produced in DES-treated groups of all YY male progeny of *O. niloticus* in the present study. This female percentage was lower than the 78% female in mixed sex progenies of several strains of *O. niloticus* reported by Mair and Santiago (1994) using the same dosages of DES (1000 mg/kg) and similar treatment period (10 days) starting after yolk sac resorption stage (10 days after fertilisation). Low female percentages of 0% (Table 5.9.), 44.8% (Table 5.8.) and 51% (Table 5.20.) were also produced in progeny derived from the crosses of YY females with

YY males in DES treatment experiments for the production YY neofemales in the present study. These results imply that feminisation of the YY genotype by oral administration of DES may be more difficult compared to other genotypes. More detailed discussion about this will be given in the next section based on the results of feminisation rates of different genotypes presented in this chapter.

Similar female percentages of 33.8±1.5% and 32.0±5.2% (a total effect of 35.4% and 50.5% with intersexes produced by DES treatment and heat treatment, respectively) were produced in DES and heat treatment groups, respectively, suggesting that heat treatment at 36°C for 21 days during the sexual differentiation can be used as successfully as hormonal sex reversal for feminisation of all YY male *O. niloticus*.

Although functional feminisation of sex-reversed YY females by heat treatment was not investigated through progeny testing in this study, Abucay *et al.* (1999) reported that heat treated sex reversed YY females are reproductively viable. Baroiller *et al.* (1995a) demonstrated that sex reversed XX males (derived from crosses of XX females with XX neomales) by heat treatment at ≥32°C sire high percentages of females when crossed the normal XX females suggesting the functional masculinisation of genetic females.

It is interesting that similar feminisation rates were obtained by DES and heat treatment on all YY male *O. niloticus*. Although a shorter estrogen treatment period of 11 days was used in the present study compared to the heat treatment period of 21 days, the temperature sensitive period appears to have a timing and duration that is similar to the hormonal sensitive period in *O. niloticus*. Similar observation was also reported in *M. menidia* (Conover and Fleisher, 1986), *O. bonairensis* (Strussman *et al.*, 1997) and *O.*

niloticus (Baroiller *et al.*, 1995a). The coincidental timing of temperature and hormonal sensitive period might result from the effect of high temperature on the action of a hormone or related enzyme during sex differentiation (Hunter and Donaldson, 1983). Direct or indirect temperature effect on the regulation of the expression of steroidogenic enzyme genes controlling the production of aromatase (the catalyst for the breakdown of androgens to estrogen) was reported by Pieau (1994), Wibbels *et al.* (1994), Crews (1996) and Crews *et al.* (1996) in thermosensitive reptiles.

A significantly higher mean intersex percentage of 18.5±2.45 was observed in heat treatment group compared 1.6±0.80 in the DES treated group and no intersex progenies were produced in any control replicates in the present study. The occurrence of intersexes in hormone treatment of fish indicates incomplete sex reversal and was also reported by Rothbard *et al.* (1981) and Mair *et al.* (1987b). However, intersex fish seem to be rare or not produced in heat manipulation experiments generally. Mair *et al.* (1990) reported 9.34%, 0.1% and 4.9% intersex in only one cross of *O. aureus*, *O. niloticus* and *O. mossambicus*, respectively, reared at low temperature (20°C). No intersex fish were observed in high temperature treatment of all YY male and putative all female progenies of *O. niloticus* by Abucay *et al.* (1999) and Baroiller (1995a,b), respectively.

From the present study, it can be concluded that high temperature treatment, which is more environmentally friendly and less harmful to human health compared to hormones, can be used for sex-reversal of all YY male progenies of *O. niloticus*. However, the low survival rate with occurrence of high numbers of intersexes may limit the use of this technique commercially.

5.4.2. Sex determination in *O. niloticus*

In the present study, YYRR and YYrr neofemales and males of *O. niloticus* were produced using both introgressive and purebred crossing approaches. The viability and fertility of the YY genotype in male and neofemale *O. niloticus* was also confirmed. Viable YY genotypes in tilapia species have been reported by Scott *et al.* (1989), Varadaraj and Pandian (1989), Mair *et al.* (1997) and Tuan *et al.* (1999). The results in this study only provide equivocal evidence for monofactorial sex determination mechanism with male heterogamety and female homogamety as suggested by previous studies (Penman, 1987; Shah, 1988; Mair *et al.*, 1991; Muller-Belecke and Horstgen-Schwark, 1995; Chapter 2 in this thesis). Although 100% male progenies were produced from the crosses involving YY broodstock from this study and androgenetic YY males from the previous study in Chapter 2 which suggests a monofactorial mode of sex determination, the inconsistent and heterogeneous sex ratios observed in progeny testing of many identified YY and XY neofemales and males suggesting that other factors may be operating.

Unexpected female percentages ranged between 0-23.1% with a mean of 9.5% and between 0-25.5% with a mean of 11.85% from the progeny testing of eleven putative YY males and eight YY neofemales, respectively. Much lower unexpected female percentages ranging between 0-20.5% with a mean of 1.1% and no female were produced in progeny testing of sixty-one YY males and twelve YY neofemales, respectively, in *O. niloticus* by Mair *et al.* (1997). Mean female percentages of 6.8% (ranging between 1.4-14.1%) and 1.9% (ranging between 0-1.5%) were reported from progeny testing of

seventeen YY males and six YY neofemales of *O. niloticus*, respectively, by Tuan (1997).

Unexpected sex ratios are not unique to crosses involving the YY genotype. For example, in single pair mating of normal females with sibling XX neomales, all female progeny were obtained in only five crosses out of the thirty-five crosses tested and the sex ratios of the others ranged between 65-99% female progeny in *O. niloticus* (Baroiller, 1996). Calhoun and Shelton (1983) observed that the sex ratio of progeny of XX neomales varied depending on the source of the females used. In this work, the proportion of female progeny from mothers that were halfsib or sibs of the XX neomales were significantly higher (99.9%) compared with the female progeny proportion (94.7%) of randomly selected females. The percentage of unexpected sex ratios could be an indication of the involvement of autosomal factor or factors other then the major sex determining genes or sex chromosome which may influence the sex differentiation in this species. This hypothesis was also supported by several authors based on sex ratios in this species (Mair *et al.*, 1991a, 1997; Wohlfarth and Wedekind, 1991; Abucay, 1999).

The presence of a single autosomal sex-modifying locus (SDL-2, two alleles, SR and sr) in gynogenetic *O. niloticus*, causing sex reversal from female to male in the homozygous condition (srsr), was postulated by Hussain *et al.* (1994) and Chapter 3 in this thesis. The existence of an autosomal recessive gene, epistatic to the major sex determining locus, was also reported in *O. aureus* by Mair *et al.* (1991b). Sarder *et al.* (in press) working on fully inbred clonal lines of *O. niloticus*, produced through gynogenesis and hormonal sex reversal, observed in one clonal line a high percentage of males was produced from the crosses of XX neomales from this line with their mitotic mother and a

control female. The authors suggested that some allele or combination of alleles at different loci fixed in this clonal line may cause sex reversal from female to male but with limited penetrance. A linkage between a single autosomal sex modifying locus (SDL-2, with SR and sr alleles) which causes sex reversal from female to male in the homozygous condition (srsr) with partial penetrance and red locus in gynogenetic *O. niloticus* was demonstrated in Chapter 3. Therefore, it is possible that there could be another sex modifying locus causing sex reversal from male to female direction. Thus, the presence of autosomal loci could be an explanation to unexpected sex ratios obtained in this study.

Both sex ratios produced by all males (XY and YY) and only XY males were heterogeneous ($\chi^2_{(31)}$=255.9; p<0.001 and $\chi^2_{(19)}$=33.9; p<0.05, respectively). Progeny sex ratios of normal XY varied from 32.5-73.1% (mean of 56.6±2.62) with a small but significant overall excess of males. This distribution is similar to but more homogeneous than that observed for Egypt-Swansea strain by Mair *et al.* (1991a and 1997), for Egypt-AIT-strain by Tuan (1997) and for Ivory coast strain of *O. niloticus* by Shelton *et al.* (1983). Mean male percentages of 56.6%, 51.8%, 52.4%, 54.7% and 50.95% were observed by this study, Mair *et al.* (1991a), Mair *et al.* (1997), Shelton *et al.* (1983) and Tuan (1997), respectively. The occurrence of slightly higher male percentages might have resulted from: i) testing of low family numbers (20); ii) strain differences; and iii) differential mortality. The observed variation in sex ratios from the present and previous studies may reflect the involvement of autosomal influences in sex determination in *O. niloticus* as suggested by Shelton *et al.* (1983), Mair *et al.* (1997) and Tuan (1997).

In the present study, progeny testing of YY males and YY neofemales from YY x YY crosses gave higher mean sex ratio of 95.5 and 100% male, respectively. A similar observation was reported by Mair *et al.* (1997). They obtained a mean sex ratio of 98.8±3.1% male in the progeny testing of YY males from crosses of YY x YY genotypes compared with a mean of 95.6±7.9 male in the progeny testing of YY male from the other crosses (XY x XY and XY x YY). The authors recommended that selection of YY males which produced progeny with sex ratios >94% male in initial progeny testing could improve the proportion of males in progeny from the crosses with normal females. The authors further claimed that the influence of autosomal sex modifying genes, operating as a threshold trait, may be the reason for the occurrence of unexpected females and selection might be a way to increase the proportion of males in the progeny of YY males. This claim was based on the influences of paternal and maternal effects on sex ratio of YY males in *O. niloticus* reported by Capili (1995), and Tuan (1997).

The effect of temperature on sex differentiation, demonstrated by several authors (See Section 5.1.2.2.) should also be considered as a further possibility in variable sex ratios in *O. niloticus*. However, it is unlikely in the present study since all the experiments were carried out under controlled experimental conditions and temperature was monitored and maintained at 28±1°C with minimal fluctuation. Differential fertilisation rates between parental genotypes and differential viability of sexual genotypes may also be possible causes of unusual sex ratios.

In the present study, the number of observed YY genotypes was lower than expected from the respective crosses in most cases. The reason for fewer YY males and YY neofemales could be differential mortality of different genotypes or differential

feminisation of the different genotypes. Although the present study was not designed to compare the feminisation rate of different genotypes, the result implies that differential feminisation of XY and YY cannot be discounted. If the female percentages from DES-treated crosses of different genotype are pooled, it can be seen that significantly lower percentages ($P<0.05$) of females (ranging between 0-51% with a mean of 24.0%) obtained in DES-treated crosses of YY x YY genotypes comparing with the female percentages of other DES-treated crosses (Fig. 5.16.): XX x YY (47.7-100% with a mean of 77.7%) XY x XY (50-96.9% with a mean of 75.3%) and XY x YY (56.8-93.2 with a mean of 83.6%).

No feminisation was achieved in DES-treated groups derived from the crosses of YYRr neofemales with androgenetic YYRR males (Table 5.17.) in the present study. According to Kirpichnikov (1981) and Yamamoto (1969) male and female genes may be located in many chromosomes and sex determination depends on a balanced effect of these genes. Therefore, male modifying genes may have been accumulated in androgenetic YYRR males due to inbreeding. The loading of autosomal male modifying genes would tend to increase in the next generation to produce YYRR neofemales by crossing YYRr neofemales with androgenetic YYRR males. It is possible that in YY progeny that carry extremely high loads of male sex modifying genes along with higher levels of endogenous androgens, feminisation could be more difficult than in other genotypes or even never takes place. The hypothesis of differential feminisation of XY

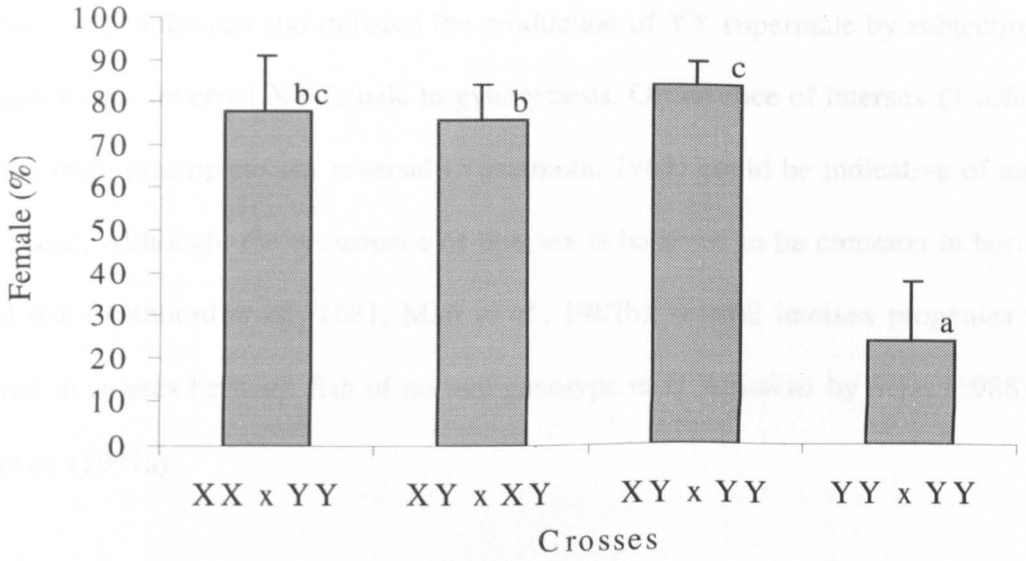


Figure 5.16. Comparison of female percentages obtained from DES-treatment of different crossing (XX x YY, n=4; XY x XY, n=5; XY x YY, n=6; YY x YY, n=4). Same superscript the bars are not significantly different.

and YY genotypes was also supported by Abucay and Mair (1997 cited in Mair *et al.*, 1997). The authors investigated the sex ratios of feminised females and nonfeminized males from DES-treated progeny of XY x YY crosses and evidence was presented for this hypothesis in one of three families.

A naturally sex reversed XX male was observed in the cross between a XY neofemale and a normal XY male which was not predicted (Table 5.13.). Occurrence of sex reversed XX male in gynogenetic *O. niloticus* resulted from an autosomal sex-modifying locus causing sex reversal from female to male was reported by Mair *et al.* (1991a), Hussain *et al.* (1994), Sarder *et al.* (in press) and Chapter 2 in this thesis. In gynogenetic common carp, similar sex reversal from female to male resulted from an autosomal recessive sex determining gene was reported by Komen *et al.* (1992).

Natural sex reversal from male to female direction was also reported by Scott *et al.* (1989) in *O. niloticus* and initiated the production of YY supermale by subjecting the spontaneous sex reversed XY female to gynogenesis. Occurrence of intersex *O. niloticus* resulting from incomplete sex reversal (Yamamota, 1969) could be indicative of natural sex reversal. Although, the occurrence of intersex is believed to be common in hormone treated fish (Rothbord *et al.*, 1981; Mair *et al.*, 1987b), several intersex progenies were observed in crosses between fish of normal genotype in *O. niloticus* by Scott (1988) and Mair *et al.* (1991a).

5.4.3. Colouration in *O. niloticus*

In the present study several homozygous (RR) and heterozygous (Rr) red Nile tilapia with different sexual genotypes were produced. Generally, the red gene acted as an autosomal locus with the red allele (R) dominant to the wild type (r) as suggested by McAndrew *et al.* (1988) and Hussain (1992). Testing of homozygous (RR) and heterozygous (Rr) individuals by crossing with wild type (rr) segregated into 1:0 (Red:wild type) and 1:1 (Red:wild type) colour ratio, respectively, according to Mendelian inheritance.

The occurrence of black blotched individuals from the cross of a XXRR female with an androgenetic YYRR male (Table 5.3.) was not predicted since homozygous red individuals normally shows no blotching or a few isolated melanistic spots around the eyes and head region (McAndrew *et al.*, 1988; Hussain, 1992). This result provides further evidence for the existence of blotching gene or genes as suggested by McAndrew *et al.* (1988). According to their hypothesis, blotching could be controlled by a single

gene with two alleles: B and b, where B is responsible for blotching and b for lack of blotching. This gene is epistatic to the red locus. If it is assumed that blotching gene is in the heterozygous state (Bb) in RR males (since two androgenetic YYRR males used in this study showed black blotching on their body surface, Plate 5.2.) and RR females , it is possible to obtain blotched and unblotched individuals from this cross. However, analysis of blotching patterns is very difficult because this trait shows a continuous nature without any obvious discrete classes. McAndrew *et al.* (1988) working on analysis of different colour genotypes of *O. niloticus* observed that the degree of blotching is reduced or increased as the amount of dominant R allele and the recessive r allele increases, respectively. However, RR individuals also show a low level of blotching whereas Rr fish display a wide range of blotching (0-24.6% of body surface). Therefore, it is possible to observe an Rr fish showing no obvious blotching, bringing difficulties for the selection of none-blotched RR homozygotes as suggested by McAndrew *et al.* (1988).

Unexpected colour segregation of 1 red: 1 wild type ratio from the crosses of RR females (RR females were originally derived from the crosses of XYRR neofemales with androgenetic YYRR males, see Table 5.7.) with rr males was produced and not predicted. This unexpected colour segregation may be the result of the involvement of an Rr female in the original cross by mistake. However, the female (000 621 331) involved in the original cross with the androgenetic YYRR male (013 296 291) was tested for colour genotype before any further crosses and was also used for production of XYRr neofemales (Table 5.11.) by crossing with a YYrr male. These results indicated that female (000 621 331) is indeed a homozygous red female. Although no plausible explanation is available for this phenomenon, it limits the use of androgenetic YYRR males for more rapid production of pure bred YYRR neofemales and YYRR males by integrating the colour and sex genotype of this males into a breeding programme before further investigation.

Chapter 6:

Summary and conclusion

6. Summary and conclusions

Research into sex determination in *O. niloticus* revealed that in this species it is predominantly monofactorial, where the female has a homogametic genotype (XX) and the male heterogametic (XY) (Penman, 1987; Shah, 1988; Mair *et al.*, 1991; Muller-Belecke and Horstgen-Schwark, 1995; Chapter 2 in this thesis). Based on the hypothesis of a single monofactorial sex determining mechanism, a model for large scale production of monosex male tilapia through the generation of the YY genotypes was proposed by Scott *et al.* (1989) and Mair *et al.* (1993). This technique has been adopted in this work in order to produce all red male *O. niloticus*. Therefore, this thesis deals mainly in the improvement in genetic control of sex ratio and colouration in *O. niloticus* through the use of genetic manipulation and controlled breeding techniques in order to produce YYRR males and YYRR neofemales. Thus, it is possible to combine red colouration and all male production in a single strain by crossing these novel YYRR males with normal XXRR females to produce genetically all red male population for tilapia growers. YYRR males and YYRR neofemales could be distributed to hatchery operators to make the production self-sustained.

Basically two options were aimed for production of YYRR males and YYRR neofemales in the present study. These were;

a) Production of YYRR males and YYRR neofemales by combining androgenesis and hormonal feminisation: Although androgenesis can be used to produce YYRR genotypes in only one generation and therefore reduces effectively the time consuming procedure of progeny testing, the result from the present study showed that the low viability of androgenetic tilapia with the potentially undesirable

efffects of inbreeding and unsuccessful feminisation of these fish were limitations to use of this technique in commercial production of YYRR genotypes.

b) Production of YYRR males and YYRR neofemales by integrating existing YYrr, YYRR and XYRR males and YYrr neofemales: Although YYRR males and YYRR neofemales can be produced starting from scratch in a strain, it will take a long time to complete such a breeding programme. In this regard, the principal question is how to combine both YY and RR genotypes in a single strain without undergoing 5 ½ generations of breeding and progeny testing which is the essential procedure to identify different sexual genotypes (Mair *et al.*, 1993) since no visible sex-linked markers or sex-specific DNA probes are available currently in this species. Therefore four breeding programmes were proposed, by integrating existing YY genotypes into RR genotypes. These were:

i) Introgression of androgenetic YYRR males and normal XXRR females (2 ½ generations): In this breeding scheme (Fig. 5.2.) YYRR neofemales were successfully produced. However, unexpected colour segregation produced by crossing these females with an XX neomale constrained the use of this programme before further research.

ii) Introgression of androgenetic YYRR males and YYrr neofemales (2 ½ generations): Although no feminisation was achieved in order to produce YYRR neofemales in second generation of this breeding scheme (Fig. 5.3.), the YYRR males produced gave high and consistent red male percentages in progeny.

iii) Introgression of YYrr males and normal XXRR females (2 ½ generations): In this breeding scheme (Fig.5.4.) no YYRR males were produced while only one YYRR neofemale was produced. However, large numbers of YYRR males and YYRR neofemales could be produced by crossing this YYRR neofemale with normal XYRR males and feminisation of some of these fry, respectively.

iv) Introgression of YYrr neofemales and normal XYRR males (3 generations): Although this breeding scheme (Fig. 5.5.) took longer than the other breeding programme described above, higher numbers of YYRR males and YYRR neofemales with more consistent and higher male percentages in progeny testing were produced compared to the YY genotypes produced from other breeding programmes. Therefore this breeding programme which is the best can be recommended for the production of YYRR males and YYRR neofemales in *O. niloticus*.

The results obtained from different experiments in this thesis should be able to offer some directions for the improvement of *O. niloticus* in terms of sex ratio and coloration. The results obtained from this thesis were summarised as follows:

1. The yield of androgenetic haploid *O. niloticus* to pigmentation stage was 24.6±3.5% (relative to controls) with optimal UV irradiation dose of 450 Jm^{-2} (at 150 μWcm^{-2}) for 5 min. The success of oocyte denuclation was assessed by using the recessive "blond" skin pigmentation character.

2. The survival rates of diploid androgenetic red *O. niloticus* was 92.84±4.30%, 10.69±2.24%, 2.03±0.60% and 0.07±0.07% (relative to controls) to morula, pigmentation, hatching and yolk sac resorption stages, respectively, using 5 min UV irradiation time at 150 μWcm^{-2} and a heat shock at 42.5°C for 3.30 min. applied at 25 minutes post fertilisation time. The survival rates reduced as development progressed. Although higher survival rates were obtained in previous studies (Myers *et al.*, 1995a; Marengoni and Onoue, 1998; see Table 2.2.) in this species, low survival rates produced in this study limit the use of this technique in the rapid production of YY males, production of clonal lines for breeding and research purposes and recovering genotypes from cryopreserved sperm.

3. A significant female but not male effect was confirmed on the survival of androgenetic *O. niloticus*, (Myers *et al.*, 1995a) suggesting that egg batches from a certain female shows different susceptibility to the UV and diploidization treatment.

4. Multilocus DNA fingerprinting produced by the hypervariable 33.15 DNA probe verified all-paternal inheritance in androgenetic *O. niloticus*. DNA fingerprinting technique can be successfully used to verify all-maternal or all-paternal inheritance in gynogenesis and androgenesis, respectively.

5. It was confirmed in this study that the YY genotype is viable and fertile in *O. niloticus* as suggested by Scott *et al.* (1989), Varadaraj and Pandian (1989), Mair *et al.* (1997) and Tuan (1999). The sex ratios of androgenetic tilapia were not significantly different from the expected sex ratio of 1 : 1 (Male : Female) indicating that *O. niloticus* has a monofactorial sex determining mechanism with female

homogamety and male heterogamety. The sex ratios produced by androgenetic males crossed to normal XX females was significantly different from a 1 : 1 sex ratio suggesting that the androgenetic male has YY genotype.

6. The mean survival rate of 19.59±3.58% meiotic gynogens (relative to controls) to yolk sac stage was produced by using UV irradiation at 250-265 μWcm^{-2} for 2 min and a heat shock at 41.5-42°C for 4 min applied at 5 minutes after fertilisation while the mean survival rate in the control groups was 33.09±3.08% in red *O. niloticus*. It can be concluded that the survival of meiotic gynogens is correlated to the survival rate of the respective control groups and is therefore probably dependent on egg quality.

7. The recombination frequencies of the red skin colour gene in meiotic gynogen *O. niloticus* produced from heterozygous red females (Rr) ranged between 0.00-0.45 with a mean of 0.12±0.04. No evidence was found for heterogeneous recombination rates or location of the red gene near a recombination hotspot suggested by Hussain *et al.* (1994).

8. All heterozygotes at the ADA*locus were produced in meiotic gynogen *O. niloticus* indicating that exactly one crossover takes place between this gene and its centromere under complete interference (where the occurrence of one crossover completely suppressed the occurrence of another). This result of this study and the previously reported results show that complete interference is common in fish species.

9. A high proportion of red but not wild type males (11%) was produced in meiotic gynogen *O. niloticus* from heterozygous red females indicating a close linkage

between a sex determining locus (SDL-2) and the red locus in this study. It appears that a recessive allele at this locus causes female to male sex reversal in the homozygous phase (srsr) but with limited penetrance. This finding could be useful for chromosome mapping of *O. niloticus*

10. There were no significant differences between colour genotypes of *O. niloticus* (namely homozygous red, heterozygous red and wild type) in terms of total fecundity, ISI (inter spawning interval), egg size and survival rate using strip-spawning methods under laboratory conditions indicating that no positive or negative pleiotropic effects of any colour morph on these reproductive traits. These results suggest that the red morph of *O. niloticus* could be used as efficiently as the wild type morph in hatchery conditions.

11. The overall mean ISI was 26.069±0.69 days and the overall mean total fecundity was 1096. A significant relationship was found between total fecundity and fish size (weight and length) in pooled data from all colour genotypes of *O. niloticus* indicating that egg number increased with increased fish size. No relationship was found between egg size and fish size. The total fecundity, relative fecundity and ISI varied widely over consecutive spawns in all colour genotypes. Overall C.V. (coefficient of variation) ranged between 7.5-78.1%, 0.3-30.9% and 1.1-52.5% in ISI, total fecundity and relative fecundity, respectively. No relationship was found between total fecundity and ISI in any colour genotype or pooled data. The results suggest that fecundity and spawning interval may be controlled by complex inter-related mechanisms involving oocyte recruitment, growth, reproductive endocrinology and individual's physiological responses to a variety of factors.

12. Similar female percentages of 33.8±1.5% and 32.0±5.2% were produced in DES and heat treated (36°C) in all YY male fry of *O. niloticus*.

13. A significantly higher intersex percentage (18.5±2.5%) was observed in heat treatment groups compared with 1.6±0.8% and 0.0% in DES treated and control groups, respectively.

14. Significantly lower survival rate of 62.9±9.8% was obtained in the heat treated groups compared to the survival rate of 97.0±0.9% in the control groups and 97.3±0.8% in the DES treatment groups. These results demonstrate that high temperature treatment could be used as an alternative option to hormones for sex reversal of all YY male progenies of *O. niloticus* because of potential risk and consumer reaction to hormone treated fish. However the low survival rate with occurrence of high intersex percentages compared to DES treatment could limit the use of this technique commercially.

15. YYRR males and YYRR neofemales were produced by integrating existing YYrr males and YYrr neofemales from Egypt-Swansea-Philippine isolate and YYRR androgenetic males from Stirling isolate with XXRR females and XYRR males Stirling isolate of Egyptian strain *O. niloticus*. These results suggest that both YY genotype and red coloration could be combined in a single strain using controlled breeding program in order to produce all homozygous red males in this strain.

16. Inconsistent progeny sex ratios were observed in progeny testing of several YY and XY males and neofemales providing unequivocal evidence for monofactorial sex determination mechanism with female homogamety and male heterogamety

suggested by previous studies. The results demonstrate that there is a strong involvement of autosomal and / or enviromental factor / factors in sex determination mechanisms in this species.

17. The low male percentages of 91.2±2.8% and 88.2±3.76% in progeny sired by YY males and YY neofemales, respectively, suggest that these fishes could not be simply used for mass production of genetically male tilapia without further refinement.

18. Progeny testing of YY males and YY neofemales from YY x YY crosses gave higher mean sex ratios of 95.5% and 100% male, respectively. The results may suggest a dosage effect of YY genotype over XY genotype resulting higher male percentages. The results also support the hypotheses that the influence of autosomal sex modifying genes, acting as a threshold trait, may be the reason for the occurrence of unexpected sex ratios in this species.

19. Significantly lower percentages of females (ranging between 0-51.0% with a mean of 24.0%) were obtained in DES treated crosses of YY x YY genotypes comparing with the female percentages of other DES-treated crosses: XX x YY (47.7-100% with a mean of 77.7%) XY x XY (50-96.9% with a mean of 75.3%) and XY x YY (56.8-93.2 with a mean of 83.6%). In addition no feminisation was achieved to produce androgenetic YYRR neofemales in *O. niloticus* in the present study. These results may suggest that feminisation of YY genotype could be difficult compared to XY genotype. Differential feminisation of different genotypes were also reported by Mair *et al.* (1997).

20. Unexpected blotched individuals were produced from the cross of an XXRR female with an androgenetic YYRR male. This result provides further evidence for the existence of a blotching gene or genes.

21. Unexpected colour segregation of 1 red : 1 wild type were obtained from the crosses of XYRR or YYRR neofemales with a XXrr neomale.

6.1. Future recommendations

Androgenesis is an important chromosome manipulation technique in terms of rapid production of YYRR genotype aimed in this present study. However low survival rate is the main limitation of this technique. A significant female effect was also confirmed on the survival of androgenetic *O. niloticus*. Therefore, selection of female parents to be used for androgenesis may improve to survival rates in this technique.

In the present study, no pleiotropic effects of colour genotypes on several reproductive traits of *O. niloticus* were found. However further research is necessary for comparison of growth performance of colour genotypes in order to assess the effects of coloration.

Several breeding programmes were applied in order to produce YYRR males and YYRR neofemales for mass production of all red males *O. niloticus*. Introgression of YYrr neofemales and normal XYRR males was found to be the most effective controlled breeding programme in terms of consistent and high male percentages. Therefore, this breeding programme could be recommended for further improvement of *O. niloticus* in terms of obtaining all red male *O. niloticus* populations over a shorter period than the 5 ½

generations required to complete the breeding programme from beginning as suggested by Mair *et al.*, 1993.

In order to consistently produce all red male tilapia, selection of YYRR males and YYRR neofemales which produce all or nearly all male progeny in initial progeny testing would be a logical step in developing the commercial YYRR male production in *O. niloticus*. To produce good stable red coloured *O. niloticus*, selection of fish showing no blotching should be carried out.

Evidence for differential feminisation of different genotypes presented in this study and previous studies should be investigated by controlled experimental conditions.

The unexpected colour segregation produced by XYRR and YYRR neofemales (originally produced from the crosses involving only RR genotypes) needs to be investigated.

It is clear that new methods must be developed in order to gain more information into the mechanism of sex determination in tilapia. Most importantly, the work on sex specific DNA probes should be carried out in order to use in time consuming progeny testing procedure.

It can be concluded that there is still work to be done in order to improve sex ratios and colouration to produce consistent results which will enhance the widespread use of all red male *O. niloticus*.

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Appendices

Appendix 1.

1.1. Tris-EDTA-NaCl (TEN) buffer for extraction of DNA

Ingredients	Amount
Tris (100 mM)	12.11 g
EDTA (10 mM)	3.72 g
NaCl (250 mM)	14.62 g

Dissolved in deionized distilled water to make the total volume 1000 ml and pH adjusted to 8.0. Buffer sterilized by autoclaving.

1.2. TBE buffer for agarose gel electrophoresis

Ingredients	Amount
Tris	108 g
Boric acid	55 g
EDTA	9.3 g

Dissolved in deionized distilled water to make the total volume 1000 ml and pH adjusted to 8.3. Buffer sterilized by autoclaving.

1.3. Saline-sodium-citrate (SSC) buffer

Ingredients	Amount
NaCl	175.3 g
Sodium citrate	88.2 g

Dissolved in deionized distilled water to make the total volume 1000 ml and pH adjusted to 7.0. Buffer sterilized by autoclaving.

Appendix 2.

2.1. Modified Fish Ringers solution (MFR)

Ingredients	Amount	Remodified chemical composition
NaCl	3.25 g	3.25 g
KCl	1.50 g	2.50 g
$NaHCO_3$	0.10 g	0.10 g
$CaCl_2.6H_2O$	0.15 g	0.15 g

Dissolved in distilled water to make the total volume 500 ml and pH adjusted to 8.0. Buffer stored at 4°C.

2.2. Cortland's saline

Ingredients	Amount
NaCl	1.81 g
$CaCl_2.2H_2O$	0.04 g
NaH_2PO_4	0.09 g
$NaHCO_3$	0.25 g
$MgSO_4$	0.06 g
Glucose	0.25 g
EDTA	0.25 g

Dissolved in 250 ml distilled water and kept at 4°C.

2.3. Tris-Borate-EDTA (TBE) buffer for starch gel electrophoresis

Ingredients	Amount
Tris (0.5 M)	60.57 g
Boric acid (0.24 M)	15.00 g
EDTA (0.016 M)	5.99 g

Dissolved in distilled water to make the total volume 1000 ml and pH adjusted to 8.5. For electrode undiluted buffer was used, for gel preparation it was diluted 1 : 10 with distilled water.

Appendix 2. (continued)

2.4. Stain (ADA*, Adenosine deaminase)

Ingredients	Amount
ADA	15 mg
MTT	5 mg
PMS	1 mg
XOD (0.025 U)	4 µl
NP (0.625 U)	10 µl

Mixed with 25 ml 0.05 M PO_4 buffer (pH 7.8) and then 25 ml 2% boiled agar (50-60°C) was added.

2.5. Fixing solution for starch gel stain

Ingredients	Amount
Acetic acid (glacial)	200 ml
Methanol	800 ml
Distilled water	1000 ml

Mixed the ingredients throughly.