GENETIC MANIPULATION STUDIES IN *OREOCHROMIS NILOTICUS* L.

A thesis presented for the degree of

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by

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Dedicated

to

My wife Koli and children Sazzad and Ali

Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged the work described in this thesis has been conducted independently and has not been submitted for any other degree.

Signature of candidate: M S Hussain

Signature of supervisor: [signature]

Date: 25/2/92

ABSTRACT

The results of a study aimed at the identification of treatment optima for triploidy induction in recently fertilised *Oreochromis niloticus* eggs by altering the intensity, duration and timing of application of pressure, heat and cold shocks are reported. Preliminary, but not directly comparable, trials suggested the following treatments to be close to the individual agent optima. Pressure: 8,000 p.s.i. 2 mins. duration applied 9 mins. after fertilisation (a.f.); heat: 41 °C, 3.5 mins. duration applied 5 mins. a.f.; cold: 9 °C, 30 mins. duration applied 7 mins. a.f. In a directly comparable trial in which the eggs of eight different females were separately exposed to the optimum shocks listed above, individual triploid yields were more variable following cold shocks and mean triploid yields were, therefore, higher following pressure and heat shock. The effects of triploidy on sexual development and maturation in female and male fish were examined in successive age groups (4 - 10 months). Significant differences in ovary weight and GSI were found between 3n and 2n females. But 3n males showed normal testis development and some of them could produce aneuploid sperm. The testis weight and GSI of such males were not significantly different compared to 2n. Comparative performance of growth, biochemical composition and endocrine profiles of normal diploids and pressure, heat and cold shocked triploids was investigated. 3n females were not siginificantly different in growth rate and proximate nutrition parameters compared to the 2n controls but were found to be functionally and endocrinologically sterile. 3n males showed no significant differences in growth or proximate nutrition parameters but were gametically sterile despite showing normal 2n endocrinological profiles and secondary sexual characteristics.

Evidence is presented for successful suppression of first cleavage in *O. niloticus* and thereby the production of mitotic gynogenetics. The optimal parameters for UV irradiation of milt were 300 - 310 $\mu W/cm^2$ for 2 min at 4 °C. The optimal pressure shock was 9000 p.s.i. for 2 mins. at 28 °C at 40 - 50 mins. a.f. and that for heat was 41 °C for 3.5 mins. at 27.5 - 30 mins. a.f. Isozyme analysis of putative mitotic gynogenetic survivors at *ADA** locus confirmed homozygosity, therefore, restoration of diploidy occurred by inhibition of first mitosis. Subsequently gene-centromere recombination frequency estimated at six enzyme loci further revealed no recombination between the respective gene and centromere at all the loci. In contrast, recombination frequency in meiotic gynogenetic progeny was 0 - 100% (mean y = 0.41) which suggesting that this probably a reflection of the relative position of the various loci to centromere on their respective chromosomes. Production of heterozygous clones of *O. niloticus* was successfully carried out by crossbreeding between viable mitotic gynogenetic female and male sibs. At the same time, homozygous clones of the fish was produced by gynogenetic reproduction (retention of 2nd polar body) using optimal pressure/heat shock treatments. The outbred (OCL) and inbred (ICL) nature of two types of clonal lines were checked and identified at *ADA** marker locus. A model for the large scale production of such clonal lines is presented. The effect of inbreeding on various phenotypic characters of two types of gynogenetics (meiotic and mitotic) in comparison to full sib controls was investigated. The coefficient of variation values of growth, meristic and all reproductive traits were lowest in control, intermediate in meiotic and highest in mitotic gynogenetic group. This study revealed that the expansion of variation in such performance traits in both type of gynogenetics was possibly the result of phenotypic expression of unmasked homozygous recessive and deleterious genes due to increasing levels of homozygosity (F value).

Mendelian mode of red body colour inheritance was studied in Egyptian red *O. niloticus* and Thai red tilapia strains. Cross-breeding between red x red and red x wild parents resulted in mostly all red coloured progenies and in some red x wild crosses progenies were also segregated into red and wild types. The F_1 red hybrids mated *inter se* and back-crossed to wild type, the progeny phenotype segregated closely into approximating the expected 3 red: 1 wild and 1 red : 1 wild ratios respectively. These results demonstrate that red body colour in two mutant strains of tilapia is controlled by a single autosomal dominant "R" gene. But both the red strains contain differing proportion of heterozygotes (Rr). In order to produce pure breeding strains, it will be important to identify heterozygotes so that the "r" gene can be culled in some way. In this case, Mendelian test-cross technique has been proposed on the light of the present study as a means of probable solution.

The possible implications of above results of genetic manipulation study in the aquaculture of *Oreochromis* spp. are discussed.

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

1.1.2 Importance of tilapia in aquaculture

The farming of tilapias is a common practice at present in aquaculture throughout several regions of the world such as Asia, China, Africa and Latin America/Caribbean. According to The Food and Agricultural Organisation of The United Nations (1978; 1987), the total world production of tilapias (*Tilapia, Sarotherodon and Oreochromis* spp.) has been increased from 197,000 t in 1977 to 561,723 t in 1987. Although a negligible proportion of this production comes from aquaculture, that proportion is increasing steadily due to recent and rapid development of fish breeding and management methodologies. In the early days of the 20th century, tilapias were wild fish in the great lakes and rivers of Africa. In central African countries, farming of tilapias in ponds was introduced after the Second World War. After that the fish were gradually spread over most of the tropical and sub-tropical areas of the world. Presently, tilapias are being cultured as warmwater foodfish in over 30 developing countries (Guerrero, 1985).

Among about 70 species of tilapia so far listed as native to the rivers of Africa (Anon, 1984a), only 16 - 23 species are suitable for culture in ponds (Huet, 1970; Balarin and Hatton, 1979). Of these, only *Tilapia* (*T. zillii* and *T. rendalli*) and *Oreochromis* (*O. mossambicus*, *O. niloticus* and *O. aureus*) species are in widespread use (Hepher and Pruginin, 1982). Due to their small size and slow growth rate, *T. zillii* and *T. rendalli* are

rarely cultured but are often used to control weed growth in irrigation canals mostly in some African and Latin American countries. Among *Oreochromis* spp., *O. aureus* is familiar for its tolerance to saline and cold water conditions; *O. mossambicus* since its introduction after 1950 to several Asian countries did not flourish and proved to be a pest, because of its ability to mature early and breed more frequently resulting in the overpopulation in ponds. As a result, the species was regarded as "nuisance fish" by producers and consumers in the tropics. Since 1970, there has been in tilapia culture renewed interest has grown in some Asian countries such as the Philippines, Thailand, Taiwan and China with the introduction of the Nile tilapia, *O. niloticus*, which is preferred by the farmers because of its desirable features for aquaculture such as faster growth, high yield, tasty flesh and ease of reproduction. Meanwhile, a red mutant *Oreochromis* strain (a hybrid between albino *O. mossambicus* x *O. niloticus*, Liao and Chang, 1983) has been developed as another promising commercial strain in addition to Nile tilapia. From that time, these fish have been recognised as a prime domesticated species for farming in a wide range of aquaculture systems from simple waste-fed fish ponds to intensive culture systems (ICLARM, 1991). Therefore, in the First International Symposium on Tilapia in Aquaculture, May, 1983, Nazareth, Israel; Drs. Liao and Chen concluded that "Tilapia is no longer an African fish but an International fish. It is believed that in the future it may become the most important fin fish in the world".

Although the important natural tilapia genetic resources are in Africa, the major culture industries at present are in Asia (Smith and Pullin, 1984). Because of declining catch and catch per unit effort in numerous inland water bodies in developing Asian countries, aquaculture is becoming increasingly attractive to government and fish farmers. With the

ever increasing need for cheap sources of protein to meet the ever increasing population in developing countries, more and more attention is being focused on small scale aquaculture. Aquaculture of tilapia and other suitable fish species is expected to play a key role in economic development in terms of providing income to fish farmers, creating more job opportunities and helping meet the nutritional needs of the people (Sevilleja, 1985).

1.1.2 Need for genetic improvement research of commercial tilapia species

Despite the popularity of tilapia in worldwide aquaculture, the main drawback of all the existing commercial strains is their precocious maturation in tropical and sub-tropical pond conditions. This leads to prolific breeding and over-crowding in grow-out systems, resulting in undesirable stunting and low yields of harvestable-size fish. In many countries, where the acceptable market size is 150 g or more and tilapias are normally grown in mixed sex culture this has become a critical problem (Guerrero, 1982).

In order to overcome this serious problem, since 1960 several methods have been proposed and developed to reduce and eliminate uncontrolled reproduction in grow-out ponds. The main goal of all these methods was to produce monosex populations of tilapias by manual separation of sexes, interspecific hybridization and masculinization using hormones.

Hand or manual sexing of tilapia by examining urinogenital papillae has been suggested and investigated by several workers (Hickling, 1963; Meschkat, 1967; Shell, 1967; Guerrero and Guerrero, 1975; cited by Guerrero, 1982). This method is time consuming, laborious and some times unreliable at the smaller fish (<10 gms.).

Interspecific hybridization of tilapias to produce all male hybrids was demonstrated first by Hickling (1960), using female *O. mossambicus* and male *O. hornorum*. Chen (1969) carried out similar crosses of these two species adding reciprocal F_1, F_2 and back-crosses. He proposed that all-male hybrids (XZ) were produced by crossing the homogametic female *O. mossambicus* (XX) and homogametic male *O. hornorum* (ZZ). Subsequent reciprocal and back-crosses resulted in sex ratios close to the expected 3:1 males:female and 1:1 male:female. However all the further crosses between F_2 hybrids did not give the expected Mendelian mode of sex ratios. After that many others also came forward to initiate similar interspecific hybridization between more supposedly homogametic species involving crosses of *O. mossambicus* x *O. niloticus* (Kuo, 1969; Majumdar and McAndrew, 1983); *O. machrochir* x *O. niloticus* (Lessent, 1968; Jalabert et al., 1971; Avtalion and Hammerman, 1978; Hammerman and Avtalion, 1979; Avtalion, 1982; Majumdar and McAndrew, 1983); *O. niloticus* x *O. aureus* (Pruginin et al., 1975; Hulata et al., 1983; Majumdar and McAndrew, 1983). However, many of these crosses did not produce the predicted 100% male offspring. The results of Majumdar and McAndrew (1983) indicate that the mechanism responsible for sex determination in hybrids is indeed variable and complicated and that a number of different alleles of different strength are operating in the tilapias as a whole. On the other hand, genetic impurity of existing tilapia strains and careless maintenance of broodstocks in poor farming conditions made more problematic the method of hybridization for mass production of all-male tilapia population.

Interspecific hybridization has proved difficult in practice and has now been replaced world-wide by direct masculinization of tilapias using hormones (Shelton et al., 1978; Guerero, 1979; Wohlfarth and Hulata, 1983; Macintosh et al., 1985; Das et al., 1987; Pandian and

Varadaraj, 1987; Guerrero and Guerrero, 1988). The results of the above authors suggest that functional sex-reversal using hormones can lead to the production of all male monosex population for tilapia aquaculture, but worries about residual levels of hormone in such fish has meant that in many countries the sale of fish which have received direct hormone treatment is prohibited (Mair, 1988). Thus the principal problem of controlling reproduction of tilapias in culture ponds still remains unsolved.

There are some other problems associated with the commercial tilapia species and many existing stocks are of poor genetic material due to i) inbreeding of stocks through poor broodstock management; ii) introgression of genes from other less desirable feral tilapia species; and iii) red strains are mostly hybrids, therefore, they do not breed true.

Genetic research of cultured fish (including tilapia) has a short history compared to that of crops and domestic animals (Pullin and Capilli, 1988). Until recently very little effort and attention have been given to genetic approaches to solve the aforementioned problems in tilapia. Therefore, applied genetic manipulation research of commercially important tilapia species potentially has a significant role to play in developing sterile populations, completely homozygous lines for selective breeding and genetic improvement of mutant strains for aquaculture. These are the subject of the present thesis. The focus is on the Nile tilapia, *Oreochromis niloticus* L., which has wide appeal throughout the tropics and is also a superb 'testbed' or model species for the development of breeding methods, through genetic research (ICLARM, 1991).

1.2 AIM AND STRUCTURE OF THE PRESENT THESIS

The genetic manipulation research carried out for this thesis was concentrated into three main areas of possible practical application in tilapia aquaculture involving the studies of triploidy, mitotic gynogenesis and body colour inheritance of red tilapia strains respectively. The structure of the present thesis is diagrammatically shown in Fig. 1.2.

Chapter 3 presents, the results of a study aimed at identification of treatment optima for triploidy induction in the recently fertilised *O. niloticus* eggs by altering intensity, duration and timing of application of hydrostatic pressure, heat and cold shocks and comparative trials following the exposure of eggs from different females for triploid yields by optimal condition of various physical shocks. Triploid individuals are expected to be functionally and endocrinologically sterile and have great potential for commercial application to replace hybridization and the use of hormones in tilapia culture. Subsequent trials were aimed at the investigation of sexual maturation and performance of growth, biochemical composition, endocrine profiles of diploids and pressure, heat and cold shocked triploids.

The next important goal of the present research was to induce gynogenesis by inhibiting the first mitotic division in *O. niloticus* eggs for the production of completely homozygous individuals in the first generation and "inbred line" or "clone" in the second. Clones have great potential for selective breeding and genetic improvement of fish stocks (Han et al., 1991). After the induction of mitotic gynogenetics as a first step in the development of

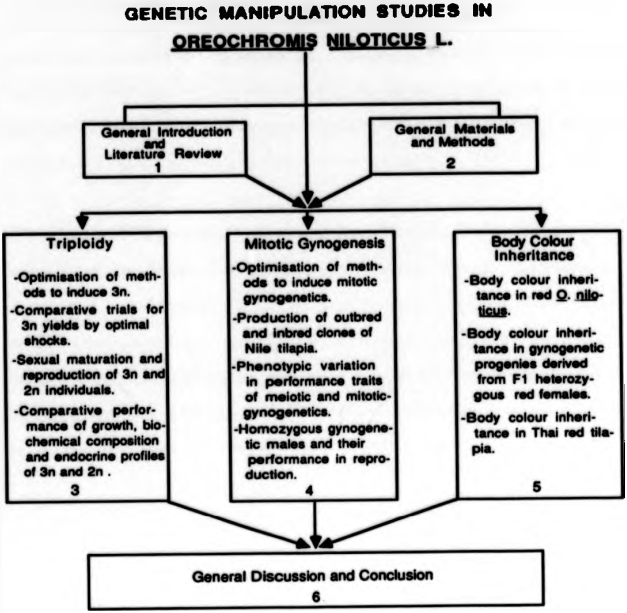

Figure 1.2. Structure of the thesis (numbers in boxes refer to thesis chapters)

clones of Nile tilapia, subsequent experiments were attemped to produce outbred and inbred clonal lines and observe the phenotypic variations in growth, meristic and various reproductive traits in meiotic and mitotic gynogenetics including the reproductive performance of homozygous gynogenetic (spontaneously sex reversed) males. Results of all these experiments are presented and discussed in Chapter 4.

Commercially available red colour strains are mostly hybrids and there is evidence that they do not breed true (Huang et al., 1988; Wohlfarth et al., 1988). The inheritance of body colour traits is complicated in such fish. Therefore, the Mendelian mode of body colour inheritance was investigated in detail in red *O. niloticus* and Thai red tilapia in order to understand the genetic bases of red body colour as suggestions on how this information may be used to produce true breeding commercial strains, desirable for aquaculture. The results of this study are described in Chapter 5.

In Chapter 6, a unified view of the results of genetic manipulation research of this thesis are outlined.

1.3 REVIEW OF LITERATURE

1.3.1 Polyploidy

Polyploidy involves the same basic principles of genome manipulation techniques as gynogenesis, except that normal sperm are used in place of irradiated sperm. This usually means the production of individuals (polyploids) with extra set(s) of chromosomes using physical or chemical treatments either to suppress the second meiotic division shortly after fertilisation of eggs (triploidy induction) or to prevent the first mitotic division shortly prior to mitotic cleavage formation (tetraploidy induction). The primary interest in polyploid induction lies in sterility of triploids and in the possibility that they could be used for growth and life expectancy instead (Purdom, 1976; Thorgaard and Gall, 1979; Gervai et al., 1980, Lincoln and Scott, 1984, Thorgaard, 1986, Nagy, 1987).

Early attempts to induce triploidy are reported in amphibians (Fankhauser and Griffiths, 1939; Fankhauser and Humphrey, 1942). In fish, the earliest reported artificial triploidization using cold shock was carried out and observed on common carp, *Cyprinus carpio* and Atlantic salmon, *Salmo salar*, respectively by Makino and Ozima (1943) and Svardson (1945). After that, Swarup (1959a; 1959b) was the first worker who successfully induced triploidy in the stickleback, *Gasterosteus aculeatus*, by cold and heat shocks and reared triploid fish up to mature stage. He was a pioneer who explained experimentally the sterility and sexual maturation conditions of triploids compared to those of diploids. In the sturgeon, *Acipenser guldenstadti*, both triploidy and tetraploidy were induced by heat shock by Vasetskii (1967; cited by Purdom, 1984).

Spontaneous triploidy among fishes has also been reported in silver crucian carp, *Carassius auratus gibelio* (Cherfas, 1966) and rainbow trout, *Oncorhynchus mykiss* (Thorgaard and Gall, 1979) and certain naturally available hybrid triploids in *Poeciliopsis* (Schultz, 1969; Cimino, 1972).

Several authors successfully crossed certain fishes and produced viable triploid progenies. These included *Pleuronectes platessa* x *Platichthys flesus* (Purdom, 1972; Lincoln, 1981); *Ctenopharyngodon idella* x *C. carpio* (Vasilyeva et al., 1975); *C. idella* x *Hypophthalmichthys molitrix* (Marian and Krasznai, 1978); *C. idella* x *Aristichthys nobilis* (Allen and Stanley, 1981; 1983; Beck and Biggers, 1982) and *O. mykiss* x *Salvelinius fontinalis* (Capanna et al., 1974; Ueda et al., 1984). Allen and Stanley (1981) reported that interspecific triploid hybrids could prove useful in fish culture because hybrid vigour and desirable attributes of both species might be combined in a relatively healthy sterile hybrid.

After the earliest success and extensive work of Swarup (1957; 1959a; 1959b), induced triploidy in fish by the retention of second polar body has been carried out by many workers. Cold shocks (Purdom, 1972; Lincoln et al., 1974; Valenti, 1975; Ojima and Makino, 1978; Wolters et al., 1982; Chourrout; 1980; Don and Avtalion, 1988a; Yamamoto and Sugawara, 1988; Baldwin et al., 1990; Manikam, 1991; Na-Nakorn, 1991); heat shocks (Chourrout, 1980; Chourrout and Quillet, 1982; Chourrout and Itskovich, 1983; Thorgaard et al., 1981; 1983; Lincoln and Scott, 1983; Utter et al., 1983; Johnstone, 1985; Bidwell et al., 1985; Don and Avtalion, 1986; 1988a; Penman et al., 1987a; Varadaraj and Pandian, 1988; 1990); hydrostatic pressure shocks (Chourrout, 1984; Lou and Purdom, 1984a; Benfey and Sutterlin, 1984a; Vasetskii et al., 1984; Allen and Myers, 1985; Benfey et al., 1988;

Lincoln, 1989); chemicals (Refstie et al., 1977; Allen and Stanley, 1979; Allen et al., 1982) and anaesthetics (Sheldon et al., 1986; Johnstone et al., 1989) have all been sucessful.

Sublethal long cold or short heat shock treatments shortly after fertilisation of eggs with normal sperm have been found to be the most convenient and easiest ways of inducing triploidy in cold and warm water fish. Higher yields of triploid individuals were obtained by cold shocks employing temperatures below 4 °C in channel catfish, *Ictalurus punctatus* (Wolters et al., 1981; 1982); bitterling, *Rhodeus ocellatus* (Ueno and Armito, 1982) and loach, *Misgurnus anguillicaudatus* (Suzuki et al., 1985a). Moderate cold shocks (5 - 15 °C) were also equally effective at producing triploid tilapia (Valenti, 1975; Don and Avtalion 1988a) and grass carp (Cassani and Caton, 1985). In salmonids, cold shocks have been found either less effective or completely unsuccessful in inducing triploidy (Lincoln et al., 1974; Lemoinie and Smith, 1980; Chourrout, 1980; Thorgaard et al., 1981; Purdom, 1969; 1983).

Nagy (1987) stated that heat shock is more effective for cold water fish rather than cold shock. Chourrout and Quillet (1982) obtained high proportions of triploid rainbow trout with survival after heat shock treatments. Short heat shocks (3 - 4 mins.) at temperatures just below the lethal level (39.5 - 42.0 °C) applied shortly after fertilisation (2.5 - 5 mins.) of eggs efficiently suppressed the second meiotic division to produce a higher percentage of triploid *Oreochromis* spp. (Chourrout and Itskovich, 1983; Penman et al., 1987a; Don and Avtalion, 1986; Varadaraj and Pandian, 1988, 1990).

Hydrostatic pressure shock was used first as an effective agent in inducing triploidy in amphibians (Dasgupta, 1962; Tompkins, 1978; Gillespie and Armstrong, 1979). Similarly in fish high pressure treatments were effective at inducing close to 100% triploids (Benfey and Sutterlin, 1984a; Chourrout, 1984; Lou and Purdom, 1984a). It was observed that pressure treatments are more effective than temperature shocks for the induction of triploidy (Benfey and Sutterlin, 1984a; Benfey et al., 1988; Johnstone, 1989; Guixiong et al., 1989). Lincoln (1989) stated that pressure shock has some advantages over heat, which uniformly affects all of the eggs giving rise to high triploid yields and lower embryo mortalities and this is not always achieved using heat because large volume of eggs impair heat transfer, which may lead to reduced triploidy rates in rainbow trout.

The primary interest in induced triploid fish involves their sterility and increased chromosome complement which may affect the phenotype and life expectancies, especially their growth and survival in mature stages (Thorgaard, 1986; Nagy, 1987). In a few studies, some authors reported an improved growth rate in triploid fish compared to diploids after maturation (Purdom, 1976; Thorgaard and Gall, 1979; Wolters et al., 1982); whereas others observed either no significant difference in juveniles (Swarup, 1957; Purdom, 1972; Cuellar and Uyeno, 1972; Gold and Avise, 1976; Gerval et al., 1980) or even inferior condition in growth rate at early stages (Penman et al., 1987a). In adult triploids, higher fillet weights and dress-out weights were recorded respectively by Lincoln (1981c) and Lincoln and Scott, 1984). Some other differences such as better feed conversion efficiencies and lower condition factors of triploid fish compared to those of diploids have been described (Wolters et al., 1982; Lincoln, 1981c; Benfey and Sutterlin, 1984b).

The the blocking of complete gametogenesis particularly in female triploids during early meiotic division results in complete inhibition of oocyte development and functional sterility (Purdom, 1972; Thorgaard and Gall, 1979; Lincoln, 1981b; Lincoln and Scott, 1984; Wolters et al., 1982; Chrisman et al., 1983; Richter et al., 1987; Nakamura et al., 1987). Despite gametic sterility of some triploid males due to meiotic inhibition of spermatogenesis, in fish species a proportion of such males are able to produce abnormal and aneuploid sperm. This ultimately leads to reproductive sterility of these males (Swarup, 1957; Lincoln, 1981a; Wolters et al., 1982; Richter et al., 1987). There is evidence that although triploid males are sterile, they become sexually mature exhibiting normal endocrine profiles and secondary sexual characteristics like normal mature diploids (Lincoln, 1981a; Lincoln and Scott, 1984; Lincoln and Bye, 1987; Benfey et al., 1987; 1989).

However, such sterility of triploid fish (both male and female) can be of benefit to aquaculture. Thorgaard (1986) and Thorgaard and Allen (1987) stated that sterility is advantageous in situations where the control of reproduction and population is desirable. Sterile grass carp were stocked for vegetation control and preventing natural reproduction in many water bodies (Wattendorf and Anderson, 1986; cited by Thorgaard et al., 1986). The use of sterile salmon triploids has been suggested for cage culture as a way to minimise gene introgression and the threat to wild stocks (Anon., 1989a; 1989b; cited by Ihssen et al., 1990). Ihssen et al. (1990) also suggested that as triploid males produce no sperm or aneuploid sperm, they could be introduced into a wild population where suppression of natural reproduction of undesirable wild female fish is required to control their

overpopulation, as any mating between the sterile triploid and any females would result in inviable eggs and a reduction in recruitment.

The mating of normal diploid and tetraploid fishes is an alternative method for producing sterile hybrid triploids. Chourrout (1984) stated that the direct production of triploids from diploid x tetraploid matings would be invaluable in species such as tilapia where the direct production of triploids from diploid females and males is limited. Therefore, a promising future of triploid fish production lies with viable tetraploid induction. It has been suggested that tetraploidy might be difficult to induce or it may be an inviable arrangement of chromosomes (Purdom, 1983). However, it appears to have been at least partially successful according to the reports of several authors in rainbow trout, *O. mykiss* (Thorgaard, 1981; Chourrout, 1984; Chourrout et al., 1986; Diter et al., 1988). Chourrout et al. (1986), who obtained fertile tetraploid male and female rainbow trout and successfully crossed tetraploid male and diploid females to produce viable triploids, although tetraploids have been produced in other species such as channel catfish, *I. punctatus* (Bidwell et al., 1985); *Oreochromis* spp (Valenti, 1985; Myers, 1986; Pandian and Vradaraj, 1987; Don and Avtalion, 1988b; Mairs, 1988), none of these authors were able to produce viable tetraploids. The aforementioned authors used various physical agents such as cold, heat and hydrostatic pressure shocks to induce tetraploidy in fish. Of the three shock treatments, pressure (6000 - 7000 p.s.i.) has been found most effective at blocking first mitotic cleavage in rainbow trout (Chourrout, 1984; Chourrout et al., 1986).

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1.3.2 Gynogenesis

Gynogenesis involves fertilising eggs with inactivated sperm and prevents any contribution of the male genome to the embryo. As a result, the embryonic development proceeds with the inheritance of only maternal chromosome set(s). Artificial gynogenesis was first reported in the frog, *Rana fusca*, by Hertwig (1911, cited by Purdom, 1983 and Ihssen et al., 1990), who demonstrated that if eggs were fertilised with sperm given increasingly higher dosage of gamma-radiation, the resulting embryos develop more normally than those fertilised with sperm irradiated with a lower dosage. This paradoxical phenomenon, which is known as "Hertwig effect" was attributed to the complete destruction of the chromosomes in the sperm at these higher dose the absence of supernumary fragments of chromosome material in same way enabling the embryos to develop as mostly inviable gynogenetic haploids. Among the survivors of haploid parthenogenetic frog embryos, some were found to be normal and viable and shown to be diploid (Parmenter, 1933) and later, it was also discovered that the frequency of normal-looking gynogenetic diploids could greatly be increased if a cold shock was applied to the eggs just after fertilisation of the amphibian eggs with irradiated sperm (Rostand, 1936; cited by Purdom, 1983; 1984).

The earliest observation of haploid gynogenesis in fish, *Salmo trutta*, was first made by Oppermann (1913), who described a typical "Hertwig effect" of massive and delayed mortalities of embryos derived from eggs fertilised respectively with partially and completely inactivated sperm. Some other workers also described an earlier study and development of parthenogenesis in Eurasian perch, *Perca fluviatilis*, and roach, *Rutilus*

rutilus (Trifonowa; 1931; 1934); shad, *Paralosa lacustris* (Lestage, 1934); northern pike, *Esox lucius* (Kasansky, 1934) and common carp, *C. carpio* (Kasansky, 1935).

Improved survival of viable gynogenetic diploids from eggs activated with X-irradiated sperm and exposed to cold shock treatments was first successfully demonstrated in common carp, *C. carpio*, loach, *Misgurnus fossilis*, and sturgeon, *Acipenser ruthenus*, by Romashov et al. (1960; 1961 & 1963). Vassileva-Dryanovska and Belcheva (1965). Purdom (1969) and Tsoy (1972) were the first to induce gynogenetic diploids by the suppression of second meiotic division of salmonid eggs.

From the reports of most of the aforementioned authors, ionizing radiation (gamma or X rays) are found to be effective for the inactivation of sperm because of their penetrating abilities which facilitates treatment of large quantities of sperm. However residual paternal characteristics or chromosome fragments may sometimes be found even after high level irradiation of sperm. It is because of this that it may not be the suitable technique for gynogenetic work (Ijiri, 1980; Onozato, 1984; Chourrout and Quillet, 1982; Thorgaard et al., 1985; Allen, 1987).

Several authors also reported the use of chemical mutagens such as diemethylsulphate, toluidine blue to inactivate the sperm in amphibians and fish (Briggs, 1952; Tsoy, 1969; Tsoy, 1972; Uwa, 1965; Chourrout, 1986). Supernumary chromosome fragments were also detected in chemical mutagen treatment of rainbow trout, *O. mykiss*, sperm but their frequency was much lower than the gamma or X rays (Chourrout, 1986).

In this respect, ultraviolet (UV) light has been found to be very suitable for sperm irradiation. Chourrout and Itskovich (1983) successfully induced gynogenesis in tilapia, *O. niloticus*, using UV irradiation of sperm and did not find any residual chromosome fragments. Similar results were also seen in other species of fishes (Stanley, 1976b; 1981; Ijiri and Egami, 1980; Chourrout, 1982; Streisinger et al., 1981; Lou and Purdom, 1984b; Taniguchi et al.,1986; 1988; Komen et al., 1988; 1991a). Thorgaard (1983) stated the low penetration of UV light, which makes it safer than ionizing rays, makes it important to have the sperm in a thin, relatively transparent film for treatment. Thick, opaque sperm samples may not be totally inactivated by UV light. Unfortunately, this makes it difficult to treat large volumes of sperm. However, the advantages of UV over other forms of irradiation are availability, transportability, ease of use and safety (Allen, 1987; Chourrout, 1987).

According to Bohm (1891; cited by Ihssen et al., 1990), a mature fish egg completes the first meiotic division before fertilisation; therefore, only the remaining cell divisions can be manipulated. Viable gynogenetic progeny can thus be produced by artificial diploidization of the maternal chromosome complement (retention of the second polar body or inhibition of first cleavage) and several physical (such as long cold shocks, short heat shocks and short hydrostatic pressure shocks) and chemical (such as antimitotics) treatments are found to be effective in fish (see Purdom, 1983; Chourrout, 1987; Nagy, 1987; and Chapter 4 of this thesis).

Long cold shocks were found to be effective for the induction of gynogenesis by interference with the meiotic spindle apparatus and extrusion of the second polar body of

fertilised eggs both in cold and warm water fish. Gynogenetic diploids in loach, *Misgurnus anguillicaudatus*, hirame, *Pralichthys olivaceus*, and common carp, *C. carpio*, were successfully induced by cold shocks (0 - 1 °C for 45 - 60 mins.) to eggs fertilised with irradiated sperm by Suzuki et al. (1985b); Tabata et al. (1986) and Komen et al. (1988). Lincoln et al. (1974) reported that cold shock below 0 °C was not effective in salmonids. In contrast shocking of < 0 °C resulted in 33% gynogenetic diploid coho salmon, *O. kisutch* (Refstie et al., 1982). Chourrout (1980) found such a level of temperature to be partly effective in diploidizing gynogenetic rainbow trout.

Moderate cold shocks (between 4 - 15 °C) were effective at inducing gynogenetic diploids in European catfish, *Silurus glanis* (Krasznai and Marian, 1987); Indian major carp, *Labeo rohita* (John et al., 1984) and paradise fish, *Macropodus opercularis* (Gervai and Csanyi, 1984). The detailed mechanism by which diploidy could be restored by cold shock after gynogenetic activation of eggs through the failure of the second, reductional phase of meiosis has been shown and described by Purdom (1983). Likewise cold shocks, short heat and hydrostatic pressure shocks have been proved to be effective in restoring viability of diploid gynogenetics in many species of amphibians and fishes. Inhibition of second polar body formation by heat shock involves the dissolution of meiotic spindle fibres and suppression of cell membrane and cell proliferation (Allen and Stanley, 1981). The high frequencies of diploid gynogenetic production by the retention of second polar body of cold water fish such as salmonids with heat shock (26 - 29 °C for 10 - 20 mins.) were reported by Chourrout (1980); Thorgaard et al. (1981); Chourrout and Quillet (1982); Refstie (1983); Purdom et al. (1985) and Chourrout (1986).

Short heat shocks of sublethal level (35 - 42 °C for 1.5 - 5 mins.) were found to be effective in inhibiting the reduction division of meiosis to diploidize the maternal genome in a number of warm water fish species such as zebra fish, *Brachydanio rerio* (Streisinger et al., 1981); common carp, *C. carpio* (Hollebecq et al., 1986; Sumantadinata et al., 1990); grass carp, *Ctenopharyngodon idella* (Cassani and Caton, 1985); Indian major carp, *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala* (John et al., 1984; 1988; Dr. M.S. Shah pers. communication); red sea bream, *Pagrus major* (Sugama et al., 1990a) and tilapias (Chourrout and Itskovich, 1983; Penman et al., 1987b; Don and Avtalion, 1988b; Mair et al., 1987; Varadaraj, 1990).

Hydrostatic pressure shock (6500 - 10,000 p.s.i. for 2 - 6 mins.) has been found to be very useful to efficiently suppress the anaphase stages of nuclear division by disruption of the metaphase spindle, resulting in diploidization of the chromosome set in eggs. The efficiency of hydrostatic pressure shock to block second polar body extrusion was first demonstrated in amphibians (Dasgupta, 1962; Tompkins, 1978; Gillespie and Armstrong, 1979; 1980; 1981). In zebra fish, Streisinger et al. (1981) successfully used pressure shocks to induce diploid gynogenesis. After that several authors reported gynogenesis using pressure shocks in salmonids (Yamazaki, 1983; Benfey and Sutterlin, 1984a; Chourrout, 1984, 1986; Lou and Purdom, 1984b; Onozato, 1984; Purdom et al., 1985); medaka, *Oryzias latipes* (Naruse et al., 1985; Ijiri, 1987); and ayu, *Plecoglossus altivelis* (Taniguchi et al., 1988).

Several authors (Benfey and Sutterlin, 1984a; Benfey et al., 1988; Johnstone, 1989; Gillespie and Armstrong, 1979) stated that among the shock treatments which prevent extrusion of the second polar body to induce diploidization or triploidization, hydrostatic

pressure shock is probably the most effective. The reason might be that pressure is less damaging to the embryos than any other physical shocks (Thorgaard, 1883; Lincoln, 1989). Lou and Purdom (1984b) observed the frequencies of diploid gynogenesis produced by hydrostatic pressure were very similar to those produced by heat shock.

The use of chemical treatments such as cytochalasin B; colchicine (Refstie et al., 1977; Allen and Stanley, 1979; Smith and Lemoine, 1979) and anaesthetics such as nitrous oxide and Freon 22 (Sheldon et al., 1986; Johnstone et al., 1989; Penman et al. in prepn.) to induce retention of the second polar body in several fishes have also been reported. Thorgaard (1983) suggested, in view of the success and ease of temperature and pressure shock treatments for inhibiting cell division in fish, chemical treatments may not be the method of choice. They are probably less adaptable to mass production than other methods.

The induction of gynogenetic diploids by artificial methods as discussed above is of real interest for their practical use in inbreeding to develop modern breeding strategies for most of the commercially important fish species (Golovinskaya, 1968; Purdom, 1969; Nagy, 1987). However, one problem still remained unsolved in the inducement of meiotic diploid gynogenesis by the retention of second polar body: the production of 100% homozygous fish. The suppression of second polar body extrusion would result in gynogenetic progeny heterozygous for many generations due to occurrence of recombination between chromatids during the first meiotic division of eggs (Purdom, 1969; Nace et al., 1970; Jaylet and Ferrier, 1978).

The analysis of recombination data of several workers (see Table 1.3) reinforces the limitations of using the method of inducing meiotic gynogenesis in fish to produce completely homozygous inbred lines. Therefore, induction of diploid gynogenesis by suppression of first cleavage at mitotic division of a zygote might be more promising method for producing inbred lines which will be homozygous at every gene locus (Chourrout, 1984; Streisinger et al., 1981).

The experimental production of completely homozygous individuals by interruption of first cleavage (endomitosis) has been carried out in many species of amphibians and mammals. Kawamura (1939) produced homozygous frogs (*R. nigromaculata*) by artificial parthenogenesis. Suppression of the first mitotic division applying pressure shock to induce homozygous diploid *Xenopus lavis* was first reported by Reindschmidt et al. (1979). Early attempts at inducing gynogenesis by the restoration of diploidy at first mitosis of eggs in plaice, *P. platessa*, were unsuccessful (Purdom, 1969). The first example of the production of viable mitotic gynogenetics in a fish, *B. rerio*, was that of Streisinger et al.(1981). In that study, fertilised eggs activated by UV irradiated sperm were treated with hydrostatic pressure or heat shocks (22.5 - 28 mins. after fertilisation) to inhibit mitotic cleavage, which resulted in 100% homozygous gynogenetic diploids. After that other workers also attempted to induce mitotic gynogenetics with limited success in fish applying late cold (Krasznai and Marian, 1987); heat (Purdom et al., 1985) and pressure (Lou and Purdom, 1984b; Onozato, 1984; Chourrout, 1984; Naruse et al., 1985) shocks. The level of temperature or pressure shocks required to suppress first mitotic cleavage is the same as or close to the level for inhibiting meiotic events. Recently, the technique has been applied successfully to common carp, *C. carpio* (Nagy, 1987; Komen et al., 1991a), medaka, *O.*

Table 1.3. Gene-centromere recombination rate estimated in gynogenetic diploids of different fishes.

Species	Loci (number)	Percent heterozygotes (average)	Authors
Carp	Morphological and proteins	8-97(35)	Cherfas (1977); Cherfas and Truweller (1978); Nagy et al. (1978; 1979); Nagy and Csanyi (1982); (cited by Thorgaard, 1983)
Carp	Morphological(2)	>90	Komen (1990)
Plaice	Enzymes(5)	18-82(44)	Purdom et al.(1976); Thompson et al. (1981); Thompson (1983); (cited by Ihssen et al. 1990)
Zebra fish	Enzyme(1) Body colour(6)	14 24-95(66)	Streisinger et al. (1981; 1986)
Paradise fish	Proteins(4)	27-66(39)	Gervai and Csanyi (1984)
Rainbow trout	Enzymes(10)	2-100(55)	Thorgaard et al. (1983)
Rainbow trout	Enzymes(8)	11-100(71)	Guyomard (1984)
Rainbow trout	Enzymes(4)	15.7-94.9(62)	Thompson and Scott (1984)
Rainbow trout	Enzymes(25)	56	Allendorf et al. (1986)
Brown trout	Enzymes(12)	60-100(88)	Guyomard (1986)
Brook trout	Enzymes(5)	6-50(33)	Fujino et al. (1989)
Brook trout	Enzymes(17)	60-100(81)	Ihssen (unpublished data, cited by Ihssen et al. 1990)
Red sea bream	Enzymes(5)	19-87(32)	Sugama et al. (1990b)

latipes (Iziri, 1987), ayu, *P. altivelis* (Taniguchi et al., 1988); Nile tilapia, *O. niloticus* (Mair et al., 1987; present work in Chapter 4 of this thesis); rainbow trout, *O. mykiss* (Quillet et al., 1991) and channel catfish, *Ictalurus punctatus* (Goudie et al., 1991).

Gynogenesis by blocking mitotic cleavage can shorten dramatically the time required to produce completely homozygous individuals in the first generation and clonal lines in the second. In contrast homozygous inbred lines can not be produced even after repeated meiotic gynogenetic reproduction or conventional methods of sib-mating for up to 10 to 20 generations (Purdom and Lincoln, 1973; Nagy and Csanyi, 1982; Han et al, 1991). Inbred lines are supposed to be very valuable products for selective breeding and improvement of fish stock (Han et al., 1991). Despite having made mitotic gynogenomes in aforementioned different fishes, until now clones have only been successfully produced in zebra fish (Streisinger et al., 1981), medaka (Naruse et al., 1985; Ijiri, 1987); common carp (Komen et al., 1991a), ayu (Han et al., 1991) and Nile tilapia (see Chapter 4 of this thesis).

1.3.3 Androgenesis

Androgenesis is a genome manipulation technique which is the reverse 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 inactivation of eggs can successfully be achieved by gamma or X-rays including UV irradiation. Androgenetic haploids have been, meanwhile, induced using gamma radiation in loach, *Misgurnus fossilis* (Romashov and Belyaeva, 1964); flounder, *P. flesus* (Purdom, 1969); salmon, *O. masou* (Arai et al., 1979); rainbow trout, *O. mykiss* (Parsons and Thorgaard, 1984; 1985) and brook trout, *Salvelinus fontinalis* (May et

al., 1988). UV light has also been reported to be successful for inactivating eggs in axolotls, *Ambystoma mexicanum* (Gillespie and Armstrong, 1980) and Nile tilapia, *Oreochromis niloticus* (Dr. J.M. Myers, pers. communication).

Stanley (1976a, cited by Ihssen et al., 1990) observed spontaneous androgenetic diploids among interspecific hybrids. A low incidence of androgenetic diploid grass carp, *C. idella*, was found among common carp female and grass carp male hybrids, which might be due to incompatibility of the two genomes resulting in elimination of the female pronucleus, producing some progeny that had only paternal inheritance. Spontaneous occurrence of androgenesis in common carp, *C. carpio*, has also been reported (Gomelsky and Recoubrastsky, 1991). Androgenetic diploid hybrids between common carp, *C. carpio*, and crucian carp, *Carassius auratus*, were produced using androgenetic technique by Grunina et al. (1991).

The first successful androgenetic diploids were experimentally induced in the Mexican axolotl shortly after fertilisation of eggs (5.5 hrs.) using heat (36 - 37 °C for 10 mins.) or hydrostatic pressure (14,000 p.s.i. for 8 mins.) shock by Gillespie and Armstrong (1980; 1981). The first androgenetic diploids in salmonids were produced by the suppression of first cleavage using pressure shock at the level of around 8500 - 9000 p.s.i. for 3 mins. (Onozato, 1982a; Parsons and Thorgaard, 1985; Scheerer, et al., 1986; May et al., 1988) and later by heat shock of 29 °C for 10 mins. (Thorgaard et al., 1990). In USSR, recently Grunina et al. (1991) were successful in inducing androgenetic common carp using late heat shock (40 - 41 °C) and producing homozygous androgenetic clones by means of second round of androgenesis utilising sperm from androgenetic males fertilised with normal eggs.

The overall rate of survival is quite low in the diploid androgenetics so far produced, this may be for a number of reasons. According to Scheerer et al. (1986), there might be a number of factors which are potentially associated in contributing to such poor viability, such as homozygosity, genotype of the sperm, damage of the egg due to irradiation, and damaging effects of treatments blocking first cleavage division. However, induction of androgenesis in fish breeding could be extremely useful in producing inbred strains and recovering genotypes from cryopreserved sperm as was pointed out by Thorgaard (1986) and Stoss (1983).

1.3.4 Inheritance of body colour in fish

After the discovery of Mendel's theory of inheritance or heredity, an immense number of experimental work has since been done by many geneticists working with various plants and animals. Meanwhile, studies have also been conducted particularly to determine the genetics of body coloration in a limited number of commercial and experimental populations of fish as well as amphibians. Goodrich and Smith (1937) were probably the first authors to study the genetics and histology of the colour pattern in the normal and albino paradise fish, *Macropodus opercularis*. Similar study on gold and blond mutations in *Lebistes reticulatus* later was followed by Goodrich et al. (1944).

The inheritance of albino body colour has been investigated in rainbow trout, *O. mykiss*, by Bridges and Limbach (1972). They observed P_1 matings normal x normal and albino x normal and the resulting progenies were all normal-coloured phenotype; albino x albino produced all albinos. When F_1 hybrids mated between themselves and back-crossed to albinos, the progenies segregated into normal and albinistic phenotypes. The frequencies

respectively were closely approximating to the expected 3:1 and 1:1 ratios, which clearly indicated that albinism in rainbow trout was a simple autosomal recessive character. Genetics and development of some colour mutations in gold fish, *Carassius auratus*, suggested that depigmentation of larval melanophores is controlled by two dominant multiple genes (Dp1 and Dp2) and albinism by double autosomal gene (p and c) and recessive transparent character by a single autosomal gene (g) (Kajishima, 1977). Wada (1990) conducted preliminary inheritance study of white (albino) coloration of the prismatic layer of shells in the Japanese pearl oyster, *Pinctada fucata martensii*, involving parental matings between white and brown strains. His results revealed that this kind of albinism in Japanese pearl oyster is inherited under the control of a recessive gene. The albino mutation is inherited as a single recessive gene in blue catfish, *Ictalurus furcatus*, and channel catfish, *I. punctatus* (Rutherford et al., 1990) and in *Rana nigromaculata* (Nishioka and Ueda, 1985).

The inheritance patterns of three mutants such as light body colour, dark (French) blue colour and white eye colour of red swamp crawfish, *Procambarus clarkii*, were determined by parental and subsequent crossbreeding trials (Black and Huner, 1980). Both light blue body colour and the white eye colour were found to transmit as recessive characters in the presence of the dominant normal body colour (red). The dark (French) blue colour phenotype appeared to be inherited as a single recessive but was sex linked with the X chromosome in females, the homozygous recessive trait in males being lethal. Genetics of golden mutation in green sunfish, *Lepomis cyanellus*, were studied by Dunham and Childers (1980) who demonstrated that the golden coloration is a recessive genetic trait.

In guppy, *Poecilia reticulata*, the body colour pattern in some mutant strains was genetically investigated by Phang and Fernando (1989) and Fernando and Phang (1990). They observed that green snakeskin having the wild-type background coloration was caused by the dominant allele (B), the yellow snakeskin being homozygous for the recessive blond allele (bb).

In carp, *Cyprinus carpio*, body colour and somatotype body form were studied involving crosses between golden and grey-coloured strains (Zhang and Pan, 1983). Golden colour seemed to be recessive to grey colour and subsequent reciprocal and back crosses indicated that body colour is controlled by 2 pairs of genetic factors. In contrast, somatype body form was considered to be a complex multigenic effect. In red grass carp, *Ctenopharyngodon idella*, induced and selective breeding experiments conducted by Tay et al. (1985) revealed that red body colour was likely to be a dominant trait.

In tilapia, a number of colour mutant strains have already been developed and their potential importance for breeding and culture has attracted considerable attention of fishery workers in many countries (see Chapter 5). Meanwhile, there are a number of reports available on genetics and inheritance of body colour in colour morphs of tilapias.

In Taiwanese red tilapia (a hybrid between mutant *Oreochromis mossambicus* x *O. niloticus*: Liao and Chen, 1983), Kuo (1969) was the first worker who reported the origin and hybridization of the fish. Later Kuo and Tsay (1984; 1985; 1986; 1987a; 1988) published five reports (in Chinese with English abstracts) on genetic improvement of such red tilapia through crossbreeding. Among their series of results, where many crosses were

made between various phenotypic individuals such as red, white, brown and wild-type: colour segregations occurred in all crosses. The results of Kou and Tsay (1984; 1988) were consistent with a partial dominance model of Taiwanese red tilapia as described by Huang et al. (1988) and Wohlfarth et al. (1990), where pink phenotype (P2P2) was found to be a homozygous dominant, red (P1P2) as heterozygote and wild-type (P1P1) a homozygous recessive. The remaining crossbreeding results (Kuo and Tsay, 1985; 1986; 1987a) are not consistent with the partial dominance model.

In the hybrid Philippine red tilapia, Galman et al. (1988) investigated breeding characteristics involving a large number of crosses between the females of different phenotypes (such as pink, grey, black spotted, albino) and the males of pink phenotype. F_1 progeny segregated closely into 75% coloured and 25% grey phenotypes. The back-crosses of females gold-orange red with males of gold-orange red and pink produced 75 - 100% coloured progenies of different phenotypes and subsequent generations of inbreeding of pink phenotype yielded 100% red, pink and albinos which indicated that pink phenotype seemed to be homozygous dominant. A similar ratio of progeny segregations in crossbreeding experiments of Philippine red tilapia strain was previously reported (Galman, 1987; Reich et al., 1990).

Behrends et al. (1982) investigated the inheritance of the red-gold colour in a mutant hybrid (*T. hornorum x T. mossambica*). They observed all red progenies in F_1 generation and segregation of both coloured (red-gold, red-brown, red-orange) and wild-type phenotypes in F_2 progenies. Inheritance of red colour in progenies derived from crosses between different colour types was more complicated than previously described. Based on the

observations of F_2 segregation, the authors stated that red-gold coloration is dominant and controlled by two or three gene pairs in this strain. Later, Behrends and Smitherman (1984) working on the same hybrid also supported the dominant mode of inheritance of red-gold body colour.

In *O. mossambicus*, genetics of red/gold body colour has been determined (Pruginin, 1987; Tave et al., 1989; Wohlfarth et al., 1990) and it was revealed that the mutant phenotype is inherited as a Mendelian recessive. Tave et al. (1989) demonstrated that black body coloured fish are homozygous dominant (GG), gold fish are homozygous recessive (gg) and bronze fish are heterozygous (Gg).

In *O. niloticus*, the mode of inheritance of body colour is controlled by a different genetic mechanism. Mires (1988) investigated this species collecting from two African sources, Uganda and Ghana and stated that the black pigmentation (normal wild colour) and the light red phenotype were respectively dominant and recessive traits. Similarly the blond mutation in *O. niloticus* has been explained as a simple Mendelian recessive by Scott et al. (1987). In a Egyptian strain of *O. niloticus*, the red body colour was inherited as an autosomal dominant trait in presence of the wild type (McAndrew et al., 1988; see also Chapter 5 of this thesis).

CHAPTER 2

GENERAL MATERIALS AND METHODS

GENERAL MATERIALS AND METHODS

2.1 FISH STOCK AND THEIR MAINTENANCE

2.1.1 Origin of fish stock

The strains of *Oreochromis niloticus* L., used in the experiments reported and discussed in this thesis came from the Tilapia Reference Collection maintained at the Institute of Aquaculture, University of Stirling, Scotland. They descended from pure stock originally obtained from a wild population in Lake Manzala, Egypt in 1979 (McAndrew and Majumdar, 1983). The Thai red tilapia, presumably a hybrid of *O. niloticus* x *O. mossambicus*, used in the body colour inheritance study was recently introduced into the Tropical Aquarium of the above Institute from Thailand in March, 1989.

2.1.2 Rearing and on-growing systems

2.1.2.1 Early fry rearing system:

The early fry rearing system used for this study consisted of a 180 litre header tank, 4 x 180 litre settling tanks, a sump tank and 4 units of rectangular rearing tanks. Each unit consisted of 14 x 15 litre perspex tanks arranged in parallel double rows of 28 tanks each side (Fig. 2.1.2a). The tanks were connected to a recirculating system, where warm water (approximately 1 litre/min./tank) came directly from the header tank by gravity into each tank through 1 cm diameter inlet pipes, the waste water was discharged through a 20 mm stand pipe into a common settling tank and through a series of biofilter tanks into the sump tank before being pumped (0.25 H.P.; Beresford Pump Ltd.) back to the header tank. The water temperature was maintained at 28±1 °C having a thermostatically controlled 3000 W

heater (Howden Ltd.) placed in the header tank.

2.1.2.2 Advanced fry rearing system:

This system consisted of 2 x 180 litre header tanks, 8 x 180 litre settling tanks, 2 x 180 litre sump tanks and a 0.75 H.P. pump and 3 units of circular rearing tanks. Two units consisted of 24 x 20-l tanks and other unit having 10 x 60 litre tanks (Fig. 2.1.2b). Each tank was equipped with an inlet pipe and a central drainage standpipe with a venturi this resulted as a circular flow of water and removal of most of the solid wastes automatically. The system was similarly connected as above to a recirculating warm water (28±1 °C) system.

2.1.2.3 Grow-out system:

This system contained 2 x 180 litre header tanks plus 1 x 115 litre overflow tank, 4 x 180 litre plus 2 x 540 litre settling tanks with biofilters and 2 x 180 litre sump tanks attached to a 0.75 H.P. pump (Beresford Pump Ltd.) . There was a total of 16 fibre glass tanks (100 cm x 100 cm x 30 cm) arranged in double row 8 tanks each on a two tier system. The tanks had valve controlled inlet pipes for incoming water and central standpipe venturi for discharging excess water and solid wastes (Fig. 2.1.2c). All the tanks were connected to a recirculating warm water (28±1 °C) system as mentioned above. Aeration was provided in each tank with one/two 15 cm air stone(s) linked to a central blower unit. Additional filtration was arranged through a stack of fine synthetic filters which received water from the overflow from the header tanks. Most of the tanks in this system were used for growth experiments as well as for all broodstock maintenance. Fish in all the above systems were maintained under 12 hour photoperiod. Water qualities such as dissolved oxygen (6.0 - 7.0

Figure 2.1.2

(a) Early fry rearing system.

(b) Advanced fry rearing system.

(c) Glass fibre tank system.

mg/l), pH (6.5 - 7.8), ammonia (0 - 0.4 mg/l) and nitrite-nitrogen (0.08 - 0.18 mg/l) and nitrate (around 20 mg/l) were regularly measured in all these systems.

2.1.3 Feeds and feeding

All fish from fry to broodstock were fed commercial trout feeds (Ewos Baker Ltd, No. 3 to 5 pellet; 40 - 50% crude protein, 15% oil, 15% carbohydrate, 10% ash, 1% fibre and 9% moisture). The early and late fry received micronised no. 3 pellet which was sieved to give a range of particle sizes (<250 micron; 250 - 500 micron; 500 - 750 micron). The fry were fed initially at 25% per body weight using the smallest particle, the size of the diet being increased (<1g 250 - 500 micron, <2g 500 - 750 micron) as the fish grew (McAndrew and Majumdar, 1989). The fry were given feeds 3 - 4 times daily, at 3 hrs. intervals. The advanced fry and fingerlings (10 - 40 g size) were fed pellet no. 3 at 3 - 7.5% body weight 3 times daily. The amount of the ration was reduced to 2 - 3% body weight and the pellet size increased as the fish grew no. 4 for 40 g up to 80 g and no. 5 for 80 + g fish and broodstock (Chipungu, 1987). The fish were not fed on the the periodical sampling days.

2.2 FISH BREEDING, STRIPPING AND FERTILISATION OF EGGS

Sexually mature and selected female brooders were transferred from broodstock holding tanks to a series of 16 glass aquaria (120 cm x 44 cm x 30 cm each Fig. 2.2) provided with recirculated, and temperature controlled (28 °C) water supply as mentioned above (section 2.1.2.1). Two to three females were accommodated in each tank but were kept separate by one or two sheets of perspex. Aeration was given in each aquarium by one/two 15 cm air stones connected to a central blower system.

Figure 2.2 Glass aquaria for tilapia breeding.

Sexually mature females of *O. niloticus* or Thai red tilapia spawn at approximately 14 - 20 day intervals under the above experimental conditions. Readiness of females to spawn was ascertained by examining the degree of swelling of the urogenital papilla and by the prespawning behaviour of the fish. The ripe female was removed from the tank and the eggs were obtained by manual stripping. The stripping was done by applying gentle downward pressure with the thumb and index fingers from just below the pectoral fins up to the genital opening of the fish. The eggs were collected in a clean and sterile Petri dish. To avoid the female prematurely releasing her eggs, she could be held in a scoop net for up to 2 hrs. while the experiment was prepared.

Immediately after stripping, the eggs could be sub-divided into a number of batches as the experimental design required. Fertilisation of eggs was carried out *in vitro* by mixing 0.1 to 0.2 ml of dry sperm or 0.4 to 0.5 ml of sperm diluted with modified Cortland's solution followed by the addition of 10 - 20 ml of 28±1 °C water. After that fertilised eggs were left in the petri dish for 2 - 3 mins. for water hardening before using for further treatment(s) or transfer to the incubation system.

2.3 APPLICATION OF SHOCK TREATMENTS

2.3.1 Application of hydrostatic pressure shock

The hydrostatic pressure shock treatments were used for optimising the inducing triploidy (see-Chapter 3) and gynogenesis (see-Chapter 4) in the relevant experiments described in this thesis. Two machines were used, which were basically identical to the commercial-scale pressure shocking device reported by Benfey et al. (1988). One was a 1.0 litre vessel kindly made available by Dr. A.G. MacDonald, Dept. of Physiology, University of Aberdeen,

Scotland and the second one a 1.5 litre vessel was designed and built by Mr. Brian Howie, Chief Engineering Technician, at the workshop of the Institute of Aquaculture, University of Stirling, Scotland (Fig. 2.3a).

To pressurize fertilised eggs, the pressure vessel and hydraulic pump reservoir were first filled with pre-warmed (28±1 °C) clean water. Eggs were held in individual uncapped vials and, after the vessel had been sealed and purged of air, the pressure release valve was closed, pressure was applied gradually by a manually operated hydraulic pump. The time taken to raise the pressure level from ambient to 8,000 - 9,000 p.s.i. was, typically, in the region of 30 secs. with the passage from 8,000 to 10,000 p.s.i. taking a further 10 secs. Pressure was released by gradually opening the valve and so the pressure dropped typically over 30 secs. (9,000 - 0 p.s.i.). After the pressure treatment, the eggs were removed from the vials and transferred directly to incubating jars described in section 2.4. The pressure loading and unloading timing was experimentally optimised. Times over 30 secs. were less effective and resulted in higher mortality.

2.3.2 Application of heat shock

For the induction of triploidy (Chapter 3) and gynogenesis (Chapter 4) in *O. niloticus*, heat shock treatments were optimised and discussed. In this section, therefore, the apparatus and basic method are detailed. A 50 litres water bath (temp. range 0 to 100 °C capable of maintaining ± 0.1 °C) equipped with a heater and stirrer (Gallenkamp Ltd) was used for heat shocking of fertilised eggs (Fig. 2.3b). About 30 minutes before the shock treatments were to be initiated, the water bath was filled with clean water and allowed to heat water up to the required temperature (typically between 39 - 42 °C). For extra accuracy, a

Figure 2.3

(a) A 1.5 litre vessel hydrostatic pressure machine deveoped at the workshop of the Institute of Aquaculture.

(b) A temperature controlled 50 litre water bath for heat or cold shock treatment.

mercury thermometer having 0.1 °C division was used to finally adjust the temperature. Fertilised eggs to be treated were first placed in netting strainer(s) floating on a bucket full of water (28±1 °C) and then the netting strainer(s) with eggs were shifted quickly into the water bath for the required duration as per design of the experiments. After the completion of heat shock, strainers containing eggs were immediately moved back to the bucket full of water (28±1 °C) or were directly transferred to incubating jars.

2.3.3 Application of cold shock

Along with pressure and heat shocks, cold shock treatments were also optimised to induce retention of second polar body in triploidy experiments described in Chapter 3. The water bath used was basically the same as above with an exception that it was fitted with an immersed spiral metallic cooling coil. The water bath having been filled with clean water was left on and adjusted to the required temperature (typically within the rage of 7 - 15 °C) by passing chilled water through the cooling coil. Fertilised eggs were similarly transferred to individual netting strainers as in heat shock treatments and exposed to cold shocking for required durations as per design of the experiments.

2.4 EGG INCUBATION AND CHECKING OF EMBRYONIC DEVELOPMENT

Fertilised, treated and untreated or control eggs were removed from the previous container and placed in netting strainer(s) floating on a bucket full of pre-warmed (28±1 °C) water. They were thoroughly cleaned and the number of eggs in each batch was estimated and recorded prior to incubation. After that the egg batches were separately and identically incubated in a series of 750 ml round bottomed plastic jars connected to the warm water (28±1 °C) recirculating system. Water in the system passed from a 125 litre header tank was

fed by gravity through a 30-W UV sterilization unit (flow rate 20l/min, UV dosage ca 62000 $\mu Wsec/cm^2$) to 34 incubating jars, each having a 1 mm diameter perspex tubing connected to the main water supply by a small airline tap to control the water flow so as to ensure gentle movement of the eggs at all times (Rana, 1986). In addition to the biofilter, this system had two shell filled filter trays to maintain pH positioned immediately above the settling tank. The survival rate of embryos in each batch was checked as per design of the experiment(s) at four development stages, morula 6-8 hrs. after fertilisation (a.f.); pigmentation 45 - 50 hrs. a.f.; hatching 80 - 90 hrs. a.f. and yolk sac resorption 10 - 12 days a.f., which was calculated as: (Number of embryos survived at a given development stages/original number of eggs) x 100.

2.5 COLLECTION, PRESERVATION AND ULTRAVIOLET IRRADIATION OF SPERM

Milt was collected by manual stripping (section 2.2), the urine was first ejected and the genital papilla dried with a paper towel and the milt sucked into a micro-pipettes by capillary attraction when it was placed at the opening of the urethra. Milt contaminated with urine or water was always eliminated. Before any milt was used for normal fertilisation in triploidy (Chapter 3) and other crossbreeding (Chapter 4 and Chapter 5) experiments motility of sperm was always examined under microscope. For short storage undiluted milt was held at 4 °C in a refrigerator and found quite viable to fertilise eggs until 3 - 4 days.

Milt samples used for UV treatment in all gynogenetic experiments described in Chapter 4 and Chapter 5 were first checked for motility and then irradiated with an ultraviolet lamp

set using a radiometer (Ultra-Violet Products Inc.). Irradiation was carried out in a 5 cm dia. Petri dish at 4 °C to give a dose of 300 - 310 $\mu W/cm^2$ for 2 mins. with a sperm concentration of 2.5 x 10^7 ml^{-1}, 2.05 ml of modified Cortland's solution (Penman et al. in prepn.).

2.6 INVESTIGATION OF CLEAVAGE

It was essential to know the exact timing of first cleavage in fertilised eggs before executing the experiment on the suppression of first mitotic division. Eggs were stripped, fertilised with normal sperm and incubated at ambient temperature (28±1 °C). The investigation was carried out from 40 - 120 mins. a.f. involving eggs from 4 different females (see-Table 2.6). A random sample of about 10 - 12 eggs were collected from the incubating jars at regular intervals (5 - 10 mins.) and immediately checked under a dissecting microscope. At 70 mins. a.f. the cleavage furrow was first observed in 10% of eggs. An intermediate timing of 90 mins. a.f. was finally found to be optimum for first cleavage in 100% of eggs. The second cleavage furrow had begun by 110 mins. a.f. (67.5% of eggs in cleavage) and it was completed by 120 mins. a.f., when 100% eggs were cleaved.

2.7 ASSESSMENT OF PLOIDY STATUS

2.7.1 Karyological examination

Karyotypes were prepared from newly hatched or one day old larvae of *O. niloticus* following modifications of published methods (Klingerman and Bloom, 1977; Chourrout and Itskovich, 1983 and Don and Avtalion, 1986). Embryos from each treatment group were placed in a small Petri dish containing 8 - 10 ml of freshly prepared (0.002 - 0.005%)

Table 2.6. Timing of first and second cleavage in *O. niloticus* eggs.

Time (mins. a.f.)	Incubation temperature (±1 °C)	Egg sample (no.±SE)	Ist cleavage (% eggs)	2nd cleavage (% eggs)
40	28	10.0±0.7	0	-
50	28	10.0±0.7	0	-
60	28	11.5±0.5	0	-
65	28	11.0±0.5	0	-
70	28	10.5±0.3	10.0± 8.7	-
75	28	10.5±0.4	47.5±23.7	-
80	28	10.5±0.4	62.5±18.8	-
85	28	11.0±0.5	97.5± 2.2	-
90	28	10.5±0.4	100	-
100	28	11.0±0.5	100	-
110	28	10.5±0.4	32.5±15.6	67.5±15.9
120	28	11.0±0.5	0	100

colchicine (Sigma Ltd.) solution for 4 -6 hrs. at 28±1 °C. Body tissues were dissected from the embryos after transfer to chilled 0.7% saline (NaCl) solution under a binocular microscope. The head and yolk sac were removed using a pair of needles and the tissues were transferred into the distilled water (hypotonic solution) for 8 - 12 minutes. The tissues were then fixed in 4:1 methanol-acetic acid, if tissues were to be stored then two further changes of fixative were given and then kept at 4 °C for a maximum of 30 - 90 days.

For slide preparation, tissues were removed from the fixative and placed, after removal of excessive fixative by blotting, in the cavity of a perspex slide with 2 - 3 drops of 60% glacial acetic acid (Analar Grade) and were minced for one minute with a glass rod to dissociate epithelial cells. After 15 - 20 minutes, 3 -4 drops of cell suspension were dropped from 30 - 40 cm height on to a clean glass slide on a hot plate (44 - 48 °C). The remaining of the drop were then sucked back within 8 -12 seconds into the micro-haematocrit dropper leaving a fine and clean ring of cells.

Slides were air dried and stained with freshly prepared 10% Giemsa stain (prepared in 0.01M phosphate buffer pH 7.0) for 15 -20 minutes. Slides were rinsed in distilled water, air dried and mounted with DPX (BDH Ltd.) after 10 minutes of xylene (BDH Ltd) wash.

Metaphase spreads of chromosomes were checked and chromosome numbers were scored by placing the slides under x400 and x1000 (oil immersion) magnifications respectively using an Olympus compound microscope. Karyological examination was assessed by counting chromosomes of several (>3) karyotypes per individual and 15 - 20 individuals per treatment group.

2.7.2 Erythrocyte nuclear measurement

Fish from each treatment group were anaesthetized (section 2.9) before collecting the blood samples. Two techniques were used to withdraw blood samples: first, in the case of younger fish not suitable for direct extraction, a sub-sample (15 - 20 individuals) was killed and the tail of each individual was severed behind the anal fin, a drop of blood was placed at one end of a slide and smeared along the slide using the edge of another slide; second, in case of larger fish blood (>0.1 ml) was extracted from the caudal vein of each fish using a 1 ml syringe and 25G hypodermic needle. A scale was removed just below the lateral line and level with the last ray of the dorsal fin, the needle was inserted at this point so as to enter the vein just below the back bone. The slides were prepared as before. Slides were air dried and stained with Wright's blood stain (Appendix 1A) for 2 mins. and then dipped in 1:1 Wright's blood stain:Sorensen's buffer (pH 6.7; Appendix 1B). Slides were rinsed in distilled water, air dried and mounted with D.P.X.(BDH Ltd.). Nuclear major axes were measured for 10 stained erythrocytes (RBC) on each slide (per fish) with an eye piece graticule at x100 magnification using an Olympus compound microscope as described by Penman et al., 1987a.

2.8 STARCH GEL ELECTROPHORESIS

Horizontal starch gel electrophoresis (McAndrew and Majumdar 1983) was used as a technique to verify the genotype of various samples from broodstock, gynogenetic and clonal line progenies. The general procedure of this technique is described in three stages: i) Sample collection and preparation, ii) Preparation of starch gel and iii) Running, slicing and staining of gels.

2.8.1 Sample collection and preparation

(a) Fin and blood samples. Caudal fin and blood samples were collected from live specimens using respectively a pair of scissors and a 1 ml syringe with hypodermic needle (section 2.7.2)). Tissues were stored separately at -20 °C until further use.

(b) Muscle, liver and kidney samples. Small pieces of skeletal muscle, the entire liver and kidney samples from freshly killed or frozen fish were taken by a scalpel and forceps. These tissues were also preserved at -20 °C until required.

For electrophoresis, tissues were taken from cold storage thawed for a few minutes and then placed in 0.5 cm diameter wells sunk into a perspex blocks. The samples were moistened with 25 µl of distilled water and homogenized using a glass rod. The crude homogenates of muscle or freeze/thaw preparation of other tissues were then absorbed onto squares of Whatman No. 1 filter paper (6x6 mm).

2.8.2 Preparation of starch gel

About 26 g of starch (Sigma Ltd.) was mixed with 220 ml of diluted TEB (22 ml buffer plus 198 ml of distilled water) or CTC (8.8 ml buffer plus 110.2 ml of distilled water) buffer (Appendix 2A)) respectively 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 a 6 mm thick gel former. The gel, covered with a glass plate, was allowed to set and cool overnight.

2.8.3 Running, slicing and staining the gel

The following morning, the gel was taken out of the former and a cut parallel to and 3 cm from the edge of the gel was made. The filter paper squares were placed along this cut with a maximum 25 samples per gel. When all samples were correctly arranged the former was placed back on the gel and a perspex spacer was (10 mm) positioned between the gel and former to keep the sample slot closed. After that the gel was placed in the electrophoretic bath with the appropriate 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. The bath tray covered with appropriate transparent lid was placed in a refrigerator at 4 °C, the power pack connected and the gel allowed to run for 3 -4 hours at 150 - 200V current.

At the end of the run the gel was removed from the bath and sample filters were removed from the gel which was then sliced horizontally into three slices, each of which could be stained for a different enzyme system (Table 2.8). The appropriate stains (Table 2.8 and Appendix 2B) for the enzyme system to be examined were weighed and mixed with stain buffer solution and 2% agar (approx. 50 - 60 °C). This 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 2C) and finally dried to seal for storage.

Table 2.8 Enzyme systems investigated in *O. niloticus*.

Enzume	Abbre-viation	E.C. No.	Buffer*	Loci no.
Adenosine deaminase	ADA	3.5.5.4	TEB	3
Esterage	EST-2	3.1.1.1	CTC	2
Aspartate aminotrans-ferase	AAT-2	2.6.1.1	TEB	2
Malic enzyme (NADP)	MEP-2	1.1.1.40	CTC	2
Aconitate hydratase	AH	4.2.1.3	TEB	2
Fumarate hydratase	FH-1	4.2.1.2	CTC	2

* TEB Tris-EDTA-Borate, pH 8.5
CTC Continuous Tris-Citrate, pH 8.0

2.9 ANAESTHESIA

In order to minimise handling stress all fish were anaesthetized using Ethyl 4-aminobenzoate (Benzocaine) at a concentration of 1 : 10,000 before all procedures (length and weight measuring, tagging, marking and blood sampling) described in different experiments. As benzocaine is not water soluble, a 10% stock solution was first prepared in ethanol and necessary concentration was made by diluting the stock solution in water at the time of sampling. The fish were immersed in the solution until opercular movement ceased, in this condition they could be handled for up to 3 minutes. After carrying out necessary sampling fish were moved to a tank or aquarium with rapid flow of clean water in order to aid their recovery from anaesthesia as well as to avoid post-sampling mortalities. Generally, fish returned to normal within a few minutes.

2.10 IDENTIFICATION OF FISH

For growth studies where fish from different treatment groups were to be stocked together in the same tank, dye marking with Alcian Blue (6% solid dye dissolved in water) was used. Each individual fish was anaesthetized (section 2.9) and marked with a treatment code on the abdomen using a pan-jet (Wilsons, Dundee). At each sampling time, marks were checked and if necessary fish were remarked.

In order to maintain individual identification all broodstock used in breeding experiments were tagged with small numbered plastic tags (Charles Neal, Finchley Ltd.). These tags were sutured through the muscle at the base of dorsal fin using a needle while the fish was anaesthetized.

2.11 FISH WEIGHING AND MEASURING

Fish weighing and measuring were essential during stocking, periodical sampling and harvesting time in all the growth experiments (Chapter 3 and Chapter 4). Weighing of fish >2 g was carried out individually after anaesthetization (section 2.9) on a digital balance ranging 0.00 - 400 g (Metler PC400, Metler Instrument AG). Fish <2 g were weighed "wet" in groups in preweighed beaker containing water in order to avoid handling mortalities. First feeding or early fry were weighed as a lot or individually after killing a sub-sample using an analytical balance (Metler H80, Metler Instrument AG). Measurements of standard length of all these fish were made using plastic callipers (Camplab Ltd.) and a scaled ruler fitted on a flat wooden frame.

2.12 HISTOLOGICAL PROCEDURE FOR GONADAL TISSUES

2.12.1 Gonad collection and fixation

Gonads (both testes and ovaries) were removed and fixed in Bouin's solution immediately after killing fish. The next day the gonadal tissues were rinsed and preserved in 70% ethanol.

2.12.2 Tissue processing

The fixed tissues were cut into appropriate sized pieces (thickness of 3-4 mm), cassetted, labelled and autoprocessed on a Histokine tissue processor (Histokinette 2000). This involved passing tissues through different alcohol grades, followed by absolute alcohol, chloroform and then impregnation in molten wax. The detailed processing schedule is given in Appendix 3. This technique worked well for testicular tissues but difficulties were found

in sectioning ovarian tissues due to over drying and incomplete impregnation of wax resulting in puffiness sections of yolky eggs. A revised protocol involving manual processing and dehydrating of ovarian tissues in an alternative alcohol series and finally in Xylene helped to overcome the previous difficulties. The manual processing of ovarian tissues was as follows: Tissues taken from 70% ethanol, similarly cut and cassetted as above and transferred to:-

85% ethanol for 2 hrs.

95% ethanol for 1 hr. (2 changes)

Ist infiltration in warm wax (60 °C)

2nd infiltration in warm wax (60 °C)

2.12.3 Embedding

When the dehydration and infiltration of both testicular and ovarian tissues were completed, they were embedded and blocked in a suitable size moulds using paraffin (55 - 60 °C) and cooled rapidly on a cold plate and finally stored at room temperature until sectioning.

2.12.4 Sectioning in microtome

The blocks were trimmed in order to bring the tissue to the surface. The blocks were then washed and cooled on a cold plate and tissues were sectioned to a thickness of 5 µm using a Leitz-Wetzlar microtome and Richert-Jung disposable microtome blades. Thin sections were floated on a water bath at 40 °C and were collected on pre-washed glass slides. The slides were then marked and dried over night in an oven (60 °C) before they were stained.

2.12.5 Staining of slides

The testicular and ovarian section slides were stained with haematoxylin and eosin. The detailed haematoxylin-eosin staining protocol for tilapia gonads is given in Appendix 3. Stained slides were finally Xylene washed for 5 mins., before mounting with DPX (BDH Ltd.).

2.13 BLOOD SAMPLING

Blood samples were taken from the caudal vein after anaesthetization (section 2.9) of fish using 21G hypodermic needle using the same technique described in section 2.7.2. The blood was allowed to clot and centrifuged at 6000 rpm for 10 mins. and the supernatant serum separated and stored at - 25 °C until further use for assaying hormone and serum calcium.

2.14 ASSAY OF TOTAL SERUM CALCIUM

Assay of total serum calcium was kindly performed by Mr. N.M. Humayun in the laboratories of Water Quality and Fish Reproduction and Genetics, Institute of Aquaculture, University of Stirling, Scotland. The atomic absorption spectrophotometry (Perkin-Elmer 2280) was used to measure total serum calcium concentration. The machine's normal range for calcium was between 0 - 4 mg/litre. To determine the correct dilution factor a dilution series was necessary for the serum samples in this machine. The serum was diluted to a series of concentration in 1% lanthanum chloride and 1% nitric acid solution. Prior to measuring the calcium of the sample, two standard solutions 2 mg/l and 4 mg/l were used for occasional calibration of the machine. Precision and reproducibility were ensured by analyzing standards each time. The serum samples were diluted to 1:250 (vol/vol) in Sterilin

tubes using the lanthanum chloride and nitric acid solution. The mixture was vortexed vigorously to ensure a homogeneous sample before measuring the reading in the atomic absorption spectrophotometer.

2.15 RADIOIMMUNOASSAY FOR 17β-OESTRADIOL AND TESTOSTERONE

Assays for 17β-oestradiol and testosterone were kindly carried out by Dr. G.P.S. Rao in the Laboratory of Fish Reproduction and Genetics, Institute of Aquaculture, University of Stirling, Scotland following the established methods of Duston and Bromage (1987).

2.15.1 Materials

a) Assay buffer:

Disodium hydrogen phosphate	8.88 g
Sodium dihydrogen phosphate	5.82 g
Sodium chloride	4.50 g
Gelatine	0.50 g
Sodium azide	0.03 g

All the above chemicals were dissolved in distilled water over a magnetic stirrer/hotplate and made up to a final volume of 500 ml.

b) Labelled hormones:

[2,4,6,7-^{3}H]Oestradiol, specific activity 85-110 Ci/mmol, and [1,2,6,7-^{3}H]Testosterone, specific activity 80-105 Ci/mmol, were obtained in 250 μCi quantities from Amersham International Ltd., Amersham, Bucks, UK. An intermediate solution was prepared by diluting 10 μl of the stock label to 2 ml with absolute ethanol. An aliquot of the intermediate solution was reconstituted in assay buffer to prepare a working solution of

approximately 20,000 dpm/100 µl (Oestradiol-17β) or 10, 000 dpm/100 µl (Testosterone).

c) Antisera:

Anti-oestradiol-17β antiserum was purchased from Steranti Research Ltd., St. Albans, Herts., UK. Anti-testosterone II (125/6) antiserum was kindly supplied by Dr. John Sumpter, Brunel University, Uxbridge, UK. The cross reaction data of these antisera with a number of structurally similar steroids are presented in Table 2.15.

Freeze-dried anti-oestradiol-17β antiserum was reconstituted with 1 ml of assay buffer and transferred in 100 µl aliquots to polystyrene tubes (LP3; Luckhams Ltd.) which were stored at -20 °C until required. The working solution was prepared by diluting one 100 µl aliquot to 10 ml with assay buffer. Freeze-dried anti-testosterone II antiserum was reconstituted in 250 µl nanopure water. A 50 µl aliquot of this solution was added to 12.45 ml nanopure water and transferred in 100 µl aliquots to polystyrene tubes (1:250 dilution) which were stored at -20 °C until required. The working solution was prepared by diluting two 100 µl aliquots to 10 ml with assay buffer.

d) Standard:

A stock standard solution of 100 ng/ml was prepared in 10 ml absolute ethanol by dissolving 1 µg dry oestradiol-17β or testosterone (both from Steranti Research Ltd.). This solution was stored at -20 °C. The working solution (10 ng/ml) was prepared freshly for each assay by diluting 100 µl stock solution to 1 ml with ethanol. Serial dilution of 100 µl aliquots of the solution provided standards of 7.8 to 1000 pg/tube for the standard curve.

e) Dextran-coated charcoal:

One 'Separex' dextran-coated charcoal tablet (Steranti Research Ltd.) was dissolved in 50 ml of assay buffer and stirred continuously on ice for 30 mins. before use.

Table 2.15. Cross reactivity of 17β-oestradiol and testosterone antisera with a number of structurally similar steroids.

	Oestradiol-17β antiserum(%)	Testosterone II antiserum(%)
Oestradiol-17β	Taken as 100	0.05
Testosterone	1.6	Taken as 100
Oestrone	7.5	0.01
Oestriol	12.2	-
11-ketotestosterone	1.0	2.4
Androstenedione	1.8	0.4
17α-hydroxy-20β-dihydroprogesterone	1.0	<0.01
17α-hydroprogesterone	1.0	<0.01
Pregnenolone	1.0	3.3
Cortisol	1.0	<0.01

(17-β oestradiol antiserum data from Duston and Bromage, 1987; Testosterone II antiserum data from Scott et al., 1984).

2.15.2 Method

a) 100 µl of each serum sample was pipetted out into separate polypropylene tubes.

b) To each tube 2 ml of ethyl acetate was added and the tubes tightly stoppered.

c) The tubes were attached to a rotary mixer for 1 hr. and then centrifuged at 1500 rpm.

d) 100 µl and 50 µl aliquots of extract were transferred to rimless soda glass assay tubes (R.B. Radley and Company Ltd., UK) for oestradiol-17β and testosterone respectively.

e) In glass assay tubes a series of dilutions were prepared covering the range of 0-1000 pg/100 µl.

f) Extracts and standards were dried in a vacuum oven below 35 °C and then dry tubes were cooled at 4 °C.

g) 100 µl of anti-oestradiol-17β or anti-testosterone antiserum was added to each tube, then 100 µl of tritiated oestradiol-17β or tritiated testosterone was also added, vortex mixed for 10 secs. and incubated overnight at 4 °C.

h) 0.5 ml of dextran-coated charcoal was added to each tube, vortex mixed and incubated for 10 mins. at 4 °C.

i) The mixture was centrifuged at 2000 rpm (4 °C) for 10 mins. and 400 µl of the supernatant (total volume 700 µl) was transferred into glass scintillation vials containing 9 ml

Optiphase 'safe' scintillation fluid (Pharmacia Ltd., Milton Keynes, Bucks., UK).

j) The vials were vortexed thoroughly and the radioactivity counted for 5 minutes in a scintillation counter (Tri-Carb 2660/2000CA; Canberra Packard). Two further vials containing 100 µl tritiated hormone were included for estimation of the total radioactivity

added to each tube and 1 vial containing scintillation fluid only for automatic subtraction of the background counts.

2.15.3 Calculations

a) The mean total dpm was multiplied by 0.4/0.7 (to correct for the difference between the total reagent volume per tube and the volume of the supernatant counted).

b) The mean non-specific binding dpm was subtracted from that of the standards and samples.

c) The percentage binding of standards and samples relative to that of the corrected total counts [% binding = (standard or sample dpm/mean total dpm) x 100] was calculated.

d) The percentage binding of the standards was plotted against the concentration of steroid in order to obtain a standard curve.

e) The concentration of the samples was read from the standard curve.

f) Multiplication was made by 0.21 (oestradiol-17β) or 0.42 (testosterone) to correct samples for volume of extract (100 or 50 µl from a total of 2.1 ml; x 21 or x 42) and volume of serum extract (100 µl; x 10) and to convert to ng/ml (x 1/1000).

2.15.4 Quality control

Ethyl acetate extracts of pooled *O. niloticus* serum were used for quality control. For extracts containing approximately 80, 20 and 6 ng/ml testosterone, the intra-assay coefficient of variation (C.V.) was 2.95, 4.34 and 16.73 % and the inter-assay C.V. was 5.01, 5.15 and 21.62 %. For extracts containing approximately 11 and 8 ng/ml oestradiol-17β, the inter-assay C.V. was 6.27 and 6.42 % and the inter-assay C.V. was 8.52 and 8.26 %.

2.15.5 Validation

Inhibition curves obtained from serial dilutions of pooled extract were parallel to the oestradiol-17β and testosterone standard curves. This indicates that the steroids detected in *O. niloticus* serum were immunologically similar to the standard hormones.

2.16 RADIOIMMUNOASSAY FOR 11-KETOTESTOSTERONE AND 11β-HYDROXYTESTOSTERONE

Assay for 11-ketotestosterone and 11β-hydroxytestosterone were kindly conducted by Dr. G.P.S. Rao in the Laboratory of the Dept. of Zoology, The University of Sheffield, UK according to the method developed by Kime and Manning (1982).

2.16.1 Materials

[1,2,6,7-^{3}H]-11-Ketotestosterone was prepared from [1,2,6,7-^{3}H]-cortisol by oxidation with chromium trioxide to androstenetrione followed by partial reduction to 11-ketotestosterone with sodium borohydrate. [1,2,6,7-^{3}H]-11β-hydroxytestosterone was prepared by oxidation of [1,2,6,7-^{3}H]-cortisol to 11β-hydroxyandrostenedione with sodium bismuthate and then partial reduction to 11β-hydroxytestosterone with sodium borohydride.

Standard steroids were obtained from Steraloids, and β-glucuronidase (from bovine liver, Type B-1) was obtained from the Sigma Chemical Company. Plastic LP3 assay tubes were purchased from Luckham. All solvents were distilled from calcium hydrate before use. Bovine serum albumin (BSA)-saline solution was prepared by dissolving 100 mg BSA in 100 ml 0.9% saline in phosphate buffer, pH 7.3.

Sodium azide (1 g/litre) was added to prevent bacterial growth. Dextran-charcoal suspension was made by mixing 750 mg of Norit A charcoal and 750 mg of dextran with 60 ml of buffered saline. A scintillation fluid containing 14 g of 2,5-diphenyloxazole (PPO) and 1.2 g of 1,4-di-[2-(5-phenyloxazolyl)]-benzene (POPOP) in 1.6 litres of toluene and 1 litre of 2-ethoxyethanol was used.

2.16.2 Preparation of antisera

The 3-(0-carboxymethyl) oxime-BSA conjugates of 11-ketotestosterone and 11β-hydroxytestosterone were prepared according to the procedure of Simpson and Wright (1977).The antisera were raised in rabbits using the procedure as described by Hargreaves and Ball, 1977. 11-ketotestosterone and 11β-hydroxytestosterone antisera were used at dilutions of 1:10,000 in BSA-saline .

2.16.3 Chromatography

Thin-layer chromatography was carried out using 20-cm^2 prepared plastic sheets of silica gel (Merk Kieselgel 60F 254) divided into 2.5 cm lanes. Androgens were separated by development in chloroform-methanol (95:5). Radioactive areas were detected using a Packard Model 7220 radiochromatogram scanner. The zones corresponding to the radioactive peaks were scraped into plastic LP3 tubes with a scalpel blade, agitated with 1 ml BSA-saline solution, and centrifuged. The supernatant was frozen until required.

2.16.4 Extraction of serum

To 100 µl of serum was added 100 µl each of [1,2,6,7-^{3}H]11-ketotestosterone and [1,2,6,7-^{3}H]11β-hydroxytestosterone solutions (0.1 µCi/ml in acetate buffer, pH 4.8). Each

radioactive solution (100 µl) was also pipetted into a counting vial to give dpm added. The serum plus radioactive tracers was extracted three times with 3 ml distilled dichloromethane, and the combined extracts evaporated to give the free steroid fraction. To the aqueous residue was added 50 µl β-glucuronidase solution (8 mg/ml in 0.1 M acetate buffer, pH 4.8) and the mixture incubated overnight. A further 50 µl enzyme solution was added and the incubation continued for a further 48 hr. Titrated steroids were added as above and the steroid moieties of the hydrolysed glucuronides extracted with dichloromethane. Both the free and glucuronide fractions were chromatographed on TLC and the two radioactive peaks corresponding to 11-ketotestosterone (R_f 0.29), and 11β-hydroxytestosterone (R_f 0.15) eluted with BSA-saline. Each eluate (100 ml) was taken for scintillation counting to measure recovery.

2.16.5 Assay of steroids

Each of the two androgens, 11-ketotestosterone and 11β-hydroxytestosterone, was assayed by the same general procedure. Tubes for the standard curve were prepared by drying a series of known weights of the steroid into plastic LP3 tubes to give suitable points on a standard curve of 0 to 400 pg. BSA-saline (100 µl) was added to each standard tube. For the serum androgen samples, 100 µl of the TLC eluate was pipetted into LP3 tubes. If high values were expected, smaller aliquots were used and the volume made up to 100 µl with BSA-saline. All samples and standards were assayed in duplicate. To each tube was added 100 µl of the appropriate steroid in BSA-saline, (approx 10,000 dpm), the tube vortexed, and allowed to equilibrate at room temperature for 30 min. The appropriate antiserum (100 µl) was added, vortexed, and left at room temperature for 30 min, then transferred to ice for 10 min. Dextran-charcoal suspension (100 µl) was added, vortexed, left in ice for 25

mins., and then centrifuged for 20 mins. at 2000 rpm. Aliquots (100 µl) from each tube were pipetted into counting vials and 10 ml ethoxyethanol scintillant added. Samples were counted for 10 mins. in a Kontron MR 300 scintillation counter fitted with dpm correction.

A standard curve of dpm vs picograms steroid was constructed from which the nanograms per millilitre of steroid in serum could be alculated for recovery.

2.17 PROXIMATE ANALYSIS OF MUSCLE SAMPLES

Proximate analysis of muscle samples was kindly conducted by Mr. N.M. Humayun in the Laboratory of Fish Nutrion, Institute of Aquaculture, University of Stirling, Scotland. According to the methods given in AOAC (1980), the proximate composition of muscle samples from different treatment groups of *O. niloticus* (Chapter 3) was determined in duplicate, sometimes in triplicate. The muscle was collected after thawing, scaling and skinning the individual fish from all the treatment groups and kept separately for subsequent analysis to observe the variation in composition among the fish belonging to different treatment groups. The moisture content of the muscle was estimated on wet weight basis and the other parameters such as crude protein, crude lipid, ash and nitrogen free extract (NFE) were analyzed on dry weight basis after drying the samples for 24 hrs. with subsequent blending to dry powder. The methods are described below:

2.17.1 Moisture

Moisture content was determined by drying a pre-weighed sample at 105 °C for about 24 hrs. in an oven.

2.17.2 Crude protein

Micro-Kjeldhal method was used to estimate crude protein by measuring nitrogen and applying the empirical factor of 6.25 (Kjeldhal factor for animal protein) to the results to convert total nitrogen to percent protein. This assumes that protein contains 16% nitrogen.

2.17.3 Crude lipid

Crude lipid was determined by extracting a weighed quantity of sample with analytical grade petroleum ether (B.P. 40 - 60 °C) using the Soxhlet apparatus. The final percent crude lipid was obtained by evaporating the residual solvent by putting the samples in an oven for 1 hr. at 105 °C.

2.17.4 Ash

Known quantities of samples from each fish were taken in dry clean porcelain crucible and then the ash content was determined by igniting the samples in a muffle furnace overnight at a temperature of 450 °C.

2.17.5 Nitrogen Free Extract (NFE)

NFE was calculated by the difference method of Hastings (1976).

2.18 SEXING AND PROGENY TESTING OF FISH

In normal *O. niloticus* broodstock and fish larger than 20 - 30 g, sex was easily differentiated by examining their urogenital papilla. The urogenital papilla in males are comparatively larger than females and consist of a single common posterior opening; on the

other hand the females have flat and shorter papilla with separate urinary and genital openings (Chervinski, 1983). Many triploid fish with undifferentiated genitalia required direct internal observation of gonads. If there was still doubt, the gonads were histologically examined to identify between ovary and testis. For progeny testing to determine the sexes in many batches of fry (<2 g size) particularly in gynogenetic experiments (Chapter 4), the manual sexing by inspecting genitalia was not useful, therefore an alternative aceto-carmine squash technique was used as described by Guerrero and Shelton (1975). In this case, a sub-sample of progenies were killed and dissected to take out the tiny thread-like gonads. Then the gonads were checked under microscope by placing them individually on clean glass slides and squashing with a cover slip over a drop of aceto-carmine stain (Appendix 4B).

CHAPTER 3

TRIPLOIDY IN NILE TILAPIA, *OREOCHROMIS NILOTICUS* L.

TRIPLOIDY IN NILE TILAPIA, *OREOCHROMIS NILOTICUS* L.

3.1 INTRODUCTION

Many agents such as physical shocks and chemicals can interfere with the normal functioning of the spindle apparatus during cell division. This can lead to the production of polyploid individuals (triploids and tetraploids) in natural populations of oviparous animals. The controlled use of these techniques has been applied to a variety of fish species in recent years (for review, see Purdom, 1983; Thorgaard, 1983; 1986; Chourrout, 1987; Ihssen et al., 1990 and section 1.3 of this thesis). Triploids are most commonly produced directly, that is, by the induction of second polar body retention during the second meiotic division of newly fertilised eggs but may also be generated indirectly by crossing of tetraploid and diploid individuals. Tetraploids may be induced by the suppression of first cleavage of the mitotic division of eggs. A schematic diagram of inducing polyploidy in fish using pressure, heat and cold shocks is given in Fig. 3.1.

Triploid individuals are expected to be sterile because of a failure of homologous chromosomes to synapse correctly during the first meiotic division and such sterility has the potential for increasing fish production because metabolic energy used for gonad development could be used for increasing growth and life expectancy instead (Purdom, 1976; Allen and Stanley, 1978; Thorgaard and Gall, 1979; Gervai, et al., 1980; Utter et al., 1983; Lincoln and Scott, 1984; Thorgaard, 1986; Thorgaard and Allen, 1987; Nagy, 1987).

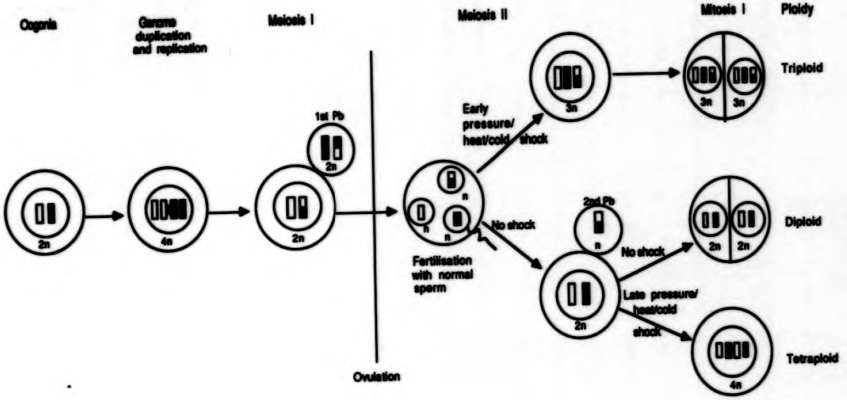


Figure 3.1. A schematic diagram of inducing polyploidy in fish using pressure, heat and cold shocks

Therefore, it was thought that triploid individuals would have a faster growth rate than diploids.

The performance of triploids in comparison to their diploid sibs seems to vary with stage of development and also seems to be species specific. During embryonic and larval development triploid *Oreochromis spp.* were significantly inferior and smaller compared to diploid sibs (Penman et al., 1987a). At juvenile stages no difference in growth rate was found between diploids and triploids in stickleback (Swarup, 1957); plaice (Purdom, 1972); rainbow trout (Cuellar and Uyeno, 1972); Californian roach (Gold and Avise, 1976) and carp (Gervai et al., 1980). In European catfish (Krasznai and Marian, 1986) and *Tilapia aurea* (Valenti, 1975) the growth rate values of triploids during their first 13 - 14 weeks were significantly higher than those of diploids.

During the spawning period of their diploid counterparts, adult triploid fish achieved more weight and became heavier (Purdom, 1976; Thorgaard and Gall, 1979; Lincoln, 1981c). In channel catfish (Wolters et al., 1982) a significantly higher growth rate of mature triploids in comparison to diploids was observed. Several workers did not find significant differences between tripoid and diploid growth rates even after the stage of sexual maturity in some fishes such as Atlantic salmon (Benfey and Sutterlin, 1984b; Benfey et al., 1889a); loach (Suzuki et al., 1985a); coho salmon (Johnson et al, 1986) and African catfish (Rickter et al., 1987; Henken et al., 1987). Experimental results with rainbow trout (Thorgaard et al., 1982; Lincoln and Scott, 1983); coho salmon (Utter et al., 1983) and tilapia (Penman et al., 1987a) suggest that induced triploids in these fishes may be somewhat less viable than diploids.

Sterile triploid fish might have some advantage over diploids where cultivation period extends into the reproductive phase of the life cycle (Woodhead, 1960 -cited by Lincoln, 1981c). Stocking of these fish into the production systems would prevent overpopulation and uncontrolled reproduction and would show at least significantly higher fillet weight expressed as dress-out percentage for the same weight of diploid counterparts (Lincoln, 1981c; Chrisman et al., 1983; Lincoln and Scott, 1984; Krasznai and Marian, 1986).

Reproductive sterility in triploid fish, provides some significant benefits in aquaculture. Triploidy apparently inhibits complete gametogenesis and prevents oocyte development in females due to failure of meiosis. As a result ovaries remain string-like and smaller at all stages of sexual development compared to those of diploids (Purdom, 1972; Thorgaard and Gall, 1979; Lincoln, 1981b; Lincoln and Scott, 1984; Wolters et al., 1982; Chrisman et al., 1983; Krasznai and Marian, 1986; Richter et al., 1987; Nakamura et al., 1987). Although in triploid female amphibians, viz. newts (Kawamura, 1951) and axolotl (Fankhauser and Humphrey, 1950), some vitellogenic eggs developed, but the embryos originating in amphibians from fertilization of such eggs with normal sperm from diploid males were not viable. Similarly in triploid *O. aureus* some vitellogenic eggs were observed (Penman 1989). As triploid females are sterile, they do not show any change of endocrine profiles and sign of secondary sexual characteristics associated with the reproductive cycle of diploids (Lincoln and Scott, 1984; Nakamura et al., 1987; Lincoln and Bye, 1987; Benfey et al., 1987; 1989a).

In contrast, in spite of functional sterility due to meiotic inhibition of spermatogenesis in most male triploid fish (Swarup, 1957; Lincoln, 1981a; Wolters et al., 1982; Richter et al.,

1987), they exhibit similar testes development, endocrine profiles and secondary sexual characteristics as mature diploid males (Lincoln, 1981a; Lincoln and Scott, 1984; Lincoln and Bye, 1987; Benfey et al., 1987; 1989a).

Tilapias (*Oreochromis, Sarotherodon* and *Tilapia spp.*) are a group of fishes of major economic importance in tropical and sub-tropical countries of the world and are widely recognized as a group of species with great potential for farming in a wide range of aquaculture systems. However, their precocious sexual maturation and fertility have profound and limiting effects on their growth. The prolific breeding results in overpopulation in mixed sex culture systems which ultimately constitutes a serious constraint on their efficient production and wider application. Although interspecific hybridization of these species leading to all male stocks has been proposed as a possible solution to this problem (Hickling 1960), it has proved difficult to maintain this practice and has now been largely replaced world-wide by direct masculinization using hormones (Shelton et al., 1978; Guerrero, 1979; Wohlfarth and Hulata, 1983; Macintosh et al., 1985; Das et al., 1987, Pandian and Varadaraj, 1987; Guerrero and Guerrero, 1988).

Production of expectedly sterile triploid *Oreochromis* spp. progeny by genome manipulation techniques has great potential and has attracted considerable attention as an alternative to hybridization and the use of hormones in tilapia culture. Until recently, triploidy has been induced using various physical shocks such as cold (Valenti, 1975; Don and Avtalion, 1988a; Varadaraj and Pandian, 1988); heat (Chourrout and Itskovich, 1983; Don and Avtalion, 1986; 1988a; Penman et al., 1987a; Pandian and Varadaraj, 1988; Varadaraj and Pandian, 1990) and hydrostatic pressure (Varadaraj and Pandian, 1988). In tilapia, there are

only two papers which compare diploid and triploid performances; one on survival, growth and maturity in *Oreochromis* spp (Penman et al., 1987a) and the other on sexual development and maturation in *O. mossambicus* (Pandian and Varadaraj, 1988). These are still less than conclusive because of the limited number of individuals used and the studies were not comprehensive.

In the present study, therefore, the aim was to compare the characteristics of the various polyploid inducing agents in a commercially important single species used in aquaculture. It was intended to optimise treatment optima for triploidy induction in newly fertilised eggs of *O. niloticus* by altering intensity, duration and timings of application of pressure, heat and cold shocks. Subsequently sexual maturation, reproduction, growth, biochemical composition and endocrine profiles were studied in triploids produced by optimal shocks and compared with their diploid sibs.

3.2 MATERIALS AND METHODS

3.2.1 Source of broodstock

The *O. niloticus* broodstock used in this study came from the Tilapia Reference Collection maintained at the Institute of Aquaculture, University of Stirling, Scotland (section 2.1.1) and attention was given for their special maintenance (section 2.1.2) and feeding (section 2.1.3).

3.2.2 Egg collection and artificial fertilization

Sufficient eggs were collected by manual stripping of ovulated females (section 2.2). Once stripped, the fertilisability of *O. niloticus* eggs falls rapidly, probably due to desiccation. Since however, some experimental series of triploidy induction trials were time consuming it was considered appropriate in some cases that eggs be obtained by sequential stripping over a period of up to 1 hour. Preliminary data suggest that this method of obtaining the eggs resulted in uniformly high fertilisation rates. Immediately after stripping, each batch of eggs (as mentioned in the following section 3.2.3) was fertilised *in vitro* (section 2.2) by mixing 0.1 to 0.2 ml of fresh milt.

3.2.3 Experimental designs for triploidy induction

3.2.3.1 Optimisation trials for triploidy induction by pressure, heat and cold shocks:

To optimise retention of second polar body of fertilised eggs for triploid induction the following parameters of pressure, heat and cold shocks (section 2.3) were used: Pressure shock intensities of 7000 - 10,000 p.s.i by 500 p.s.i. steps administered for various durations

at various timings after fertilisation (see- Table 3.3.1.1); Heat shocks of 37 - 42 °C by 1 °C steps applied for various durations at various timings after fertilisation (see- Table 3.3.1.2); Cold shocks of 7 -11 °C by 2 °C steps and 15 °C applied for various durations at various timings after fertilisation (see- Table 3.3.1.3). Each treatment batch containing around 100 eggs as exposed to elevated pressure, heat and cold shocks as per design of the experiment. Control (untreated) batches of eggs were taken pooled from each sequential stripping and incubated separately for comparative purposes. A total of 39 female fishes were used in the optimisation trials, some females were used more than once but in different experimental series, the superscripts in Table 3.3.1.1, Table 3.3.1.2 and Table 3.3.1.3 show the number of females and is also equal to the number of replicates used for that particular treatment.

Initially, 7000 p.s.i. pressure shock was administered in the period between 5 - 7 mins. after fertilisation (a.f.) for 2 mins., which did not show any effect in inducing triploidy. Therefore, in the search for treatment optima, it was essential to establish a single parameter. Thus, having first established the optimum time a.f. for shock initiation at one presumed effective intensity (8000 p.s.i.) and duration (2 mins.) the direction of trials was moved towards establishing the most effective intensity and duration of shock using several females. Since, for pressure, the change from one intensity level to another was not greatly rate limiting to the performance of the trials, it was possible, in any one working day and without prejudice to the condition of the eggs, whether stripped or not, to test all the variables of importance. This was adjudged not to be the case for the temperature shock experiments, since it took some considerable time to ensure that the temperature was sufficiently stabilised at any new working level. Thus different trials were conducted on different days using, necessarily, different females. The results to determine treatment

optima, triploid rate and triploid yield figures are presented relative to the appropriate control values of the female used in separate experiment. This would tend to mask inter-individual differences in response, as not all eggs from the same female were necessarily given all treatments.

3.2.3.2 Comparative trials for triploid yields by optimal shock treatments:

The extent of any inter-individual variation in response must be the subject of a separate experiment once apparent optima had been identified. To do this the eggs of 8 different females were taken, fertilised and then divided into four lots. Three groups were taken and separately exposed to the preferred pressure (8000 p.s.i., 2 mins. duration applied 9 mins. a.f.), heat (41 °C, 3.5 mins. duration applied 5 mins. a.f.) and cold (9 °C, 30 mins. duration applied 7 mins. a.f.) shocks as identified in the earlier part of this study, the fourth group being maintained as a control. In this comparative trial essentially similar, but necessarily different numbers of eggs (80 - 200), were taken for treatment from the different females.

3.2.3.3 Production of triploidy for sexual maturation and reproduction trials:

Eggs stripped from a female fish were fertilized with fresh sperm and were then divided into three equal batches. The first and second batches of eggs were exposed to optimal pressure (8000 p.s.i for 2 mins applied 9 mins a.f.) and heat shocks (41 °C for 3.5 mins. applied 5 mins. a.f.) respectively. The third batch of eggs was used as a control.

3.2.3.4 Production of triploidy for comparative performance trials:

As described above (section 3.2.3.2), fertilised eggs of a female were divided into 4 equal

batches. Three of them were exposed separately to optimal pressure, heat and cold shocks. The fourth batch of eggs was left as control (untreated).

3.2.4 Egg incubation and survival rates

All treated and untreated (control) batches of eggs were separately incubated (section 2.4) and survival rate (S) of embryos at three development stages (morula, pigmentation and hatching, detailed in section 2.4) was calculated as (number of embryos surviving at a given development stage/original number of eggs) x 100.

3.2.5 Identification of ploidy

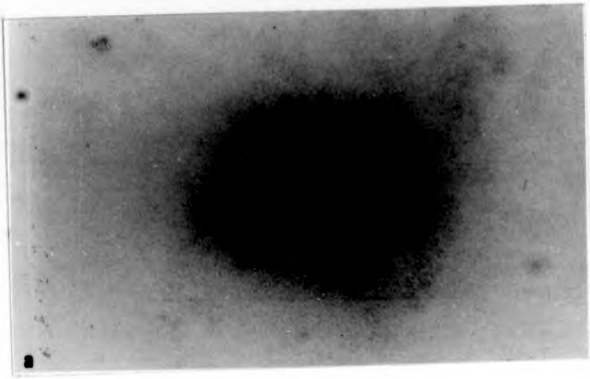
The ploidy of all treatment and control batches was determined by chromosome preparation (section 2.7.1) from a sub sample of hatched or one day old larvae. *O. niloticus* triploid metaphases are composed of three set of chromosomes (N=66) characterised by the presence of three rather than two large, easily identified, submetacentric chromosomes or marker chromosomes (Fig. 3.2.5). The triploid rate was determined by the karyological analysis of several (>3) karyotypes per individual and 15 - 20 individuals per treatment. The triploid yield of each batch was the product of the survival rate to hatch (relative to the control adjusted to 100%) and the observed triploid rate and was expressed as a percentage of the eggs originally present (Johnstone, 1985).

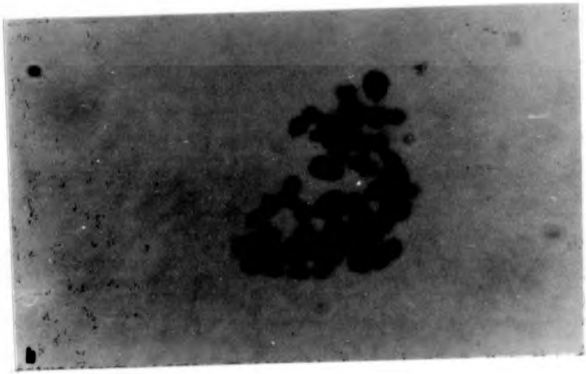
In addition to the chromosome preparation ploidy status of most of the surviving progenies induced by various optimal shock treatments was further confirmed before and after stocking for on-growing related to all proposed trials of the present study using erythrocyte nuclear measurement by estimating nuclear major axis (section 2.7.2).

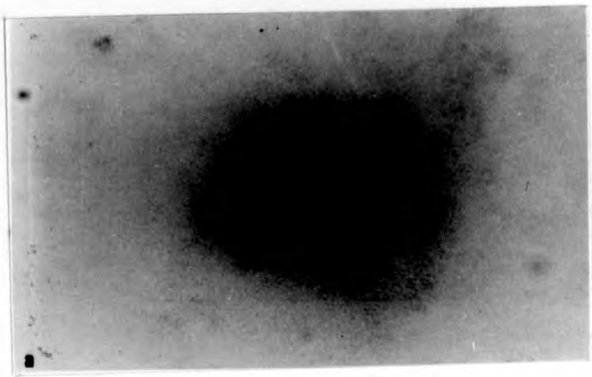
Figure 3.2.5

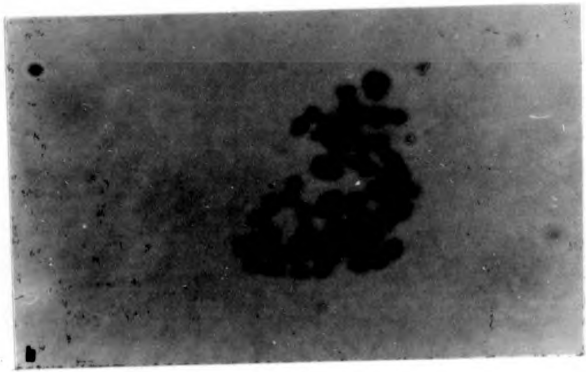
(a) Metaphase chromosomes of diploid (2n = 44) *O. niloticus*.

(b) Metaphase chromosomes of triploid (3n = 66) *O. niloticus*.









3.2.6 Fry rearing, on-growing and growth estimation

3.2.6.1 Sexual maturation and reproduction trials:

The first feeding triploid and full-sib diploid fry (10 - 12 days a.f.) were transferred from incubating jars into 20-l tanks provided with a recirculated and controlled temperature (28±1 °C) water supply (section 2.1.2). Triploids and diploids were kept separately at a stocking density of 100 fry/tank for 5 weeks. Triploid fry were grown in two separate tanks, as no differences in triploid treatment groups was noted, triploid sib fry from two shock treatments (pressure and heat) were stocked (1:1 ratio) together in one tank and only heat shocked fry in other tank. At the end of the initial rearing period, the triploid (mean weight 0.35±0.23 g) and diploid (mean weight 0.35±0.13 g) fry were stocked as before into three 100 cm x 100 cm x 30 cm fibre glass tanks at a density of 70 fish/tank and kept on 12 hr photo-period in a recirculated system maintained at 28 °C (section 2.1.2). The early fry were fed initially 25% per body weight 3 - 4 times daily using various size of ground feed particles, and advanced fry and on-growing fish were fed two - three times daily at 2 - 7 % per body weight with various sizes of trout pellets (section 2.1.3).

Monthly estimates of standard length and body weight for growth determination (section 2.11) and gonad weight for male and female triploid and diploid (5 fish/sex/treatment) were made on random sub-samples of total fish in each category from 4 months of age to the end of the growing period (10 months). The values for gonadosomatic index (GSI) were calculated as indicated below.

3.2.6.2 Comparative growth performance trials:

The first feeding fry of full-sib diploid control (2n Cont.), triploid pressure shock (3n P.S.), triploid heat shock (3n H.S.) and triploid cold shock (3n C.S.) were stocked separately into 20-l tanks at a density of 60 individuals/tank for 9 weeks. At the end of the initial rearing period 48 fingerlings from each batch were dye marked (section 2.10) individually for identification and divided into four replicated groups and restocked separately into four 100 cm x 100 cm x 30 cm fibre glass tanks. Each tank containing equal number of fish (N=12) from each treatment groups (Total stock =4 8/tank) and left for on-growing until 45 weeks. All experimental tanks were exposed to the same photoperiod and in the same recirculated water system (section 2.1.2). The fish were fed twice daily 2 - 3 % per body weight with similar type of trout feeds (section 2.1.3) as above.

Estimation of growth (section 2.11) by weight and length (standard length) was carried out once every 5 weeks from 10 weeks to 40 weeks of age for a mixed sex group of fish in experimental tanks. At the time of termination of the experiment, all the fish were killed, many triploid fish with undifferentiated genital papilla required dissection for determining sex ratios by internal observation of gonads. In total 30 fish consisted of 15 females and 15 males from each ploidy group were randomly chosen, weight and length were individually measured and individually dissected to record the weight of various body organs for calculating the following growth parameters:

Gonadosomatic index (GSI) = (gonad wt./total fish wt.)x 100

Percentage dress-out = (gutted fish wt./total fish wt.)x 100

Hepatosomatic index (HSI) = (liver wt./total fish wt.)x 100

Condition factor (K) = (gutted fish wt./standard length^3) x100

3.2.7 Gonadal histology

Ovarian and testicular samples were obtained every month (4 months to 10 months of age) and fixed in Bouin's fluid immediately after killing (5 fish/sex/treatment). Next day the samples were fixed in 70% alcohol. Dehydrating, paraffin embedding and sectioning and staining of gonad samples were made according to the recommended histological procedures (section 2.12). General histological examination was carried out under x63, x100, x250, x400 and x1000 (oil immersion) magnifications and photographed through an Orthomat Photomicroscope.

3.2.8 Crosses between triploid males and diploid females

A total of 10 crosses between triploid males and diploid females were undertaken, some males and some females were used more than once but in different crosses. Triploid males (N=8) used in these trials were 10 months of age (collected from a growout tank stocked with pressure and heat shocked triploids for a sexual maturation study). Before using for reproduction their ploidy status was confirmed by erythrocyte nuclear length measurement (section 2.7.2.). Diploid females (N=8) and males (N=8) were from the same age group and were sibs of the triploids. All milt from sexually ripe triploid and diploid males was checked under a microscope to assess the motility and concentration of the sperm. Eggs collected from a normal female were divided into two batches and fertilised, one with the triploid and other with diploid sperm. The survival rate of embryos in all the crosses was estimated at four developmental stages (section 2.4) viz. morula (MOR), pigmentation (PIG), hatch (HAT) and yolk sac resorption (YSR). The numbers of normal and deformed fry at HAT

and YSR stages were also recorded and about 15 - 20 newly hatched larvae/cross/treatment were collected and used for karyological analysis (section 2.7.1).

3.2.9 Total serum calcium determination

Blood samples (around 1.0 ml) were collected from the caudal vein (section 2.13) under anaesthesia (section 2.9) from diploid and triploid fish belonging to the 6, 7 and 8 months of age groups (5 fish/sex/treatment). This was also done for the comparative growth trial fish (7 individuals/sex/ploidy group). Blood clotting, centrifuging, separating serum and storage of the serum samples was carried out according to the procedures already described (section 2.13). Total serum calcium level of each individual fish was measured following the protocols (section 2.14) for atomic absorption spectrophotometry (Perkin-Elmer 2280). During blood sampling body weight and gonad weight of all fish of either sex were recorded to calculate the gonadosomatic index as indicated above.

3.2.10 Steroid hormone determination

Blood samples (section 2.13) were taken for selected sex steroid hormone determination from diploid and triploid fish of 6, 7 and 8 months of age groups (5 fish/sex/treatment) as well as from diploid control and pressure, heat and cold shocked triploid groups (10 fish/sex/ploidy group) at 45 weeks of age. Individual body weight and gonad weight of female and male fish of all groups were noted for calculation of GSI. Serum samples were analysed to determine the level of 17β-oestradiol and testosterone using radioimmunoassay methods (section 2.15) of Dustson and Bromage (1987). Assays for 11-ketostestosterone and 11β-hydroxytestosterone were carried out according to the methods (section 2.16) developed by Kime and Manning (1982).

3.2.11 Biochemical composition analysis

To determine biochemical composition viz. crude protein, crude lipid, moisture and ash, the muscle samples were collected by killing female and male diploid control and pressure, heat and cold shocked triploid fish (5 individuals/sex/ploidy group) at 45 weeks of age. For calculating GSI, body weight and gonad weight of either sex were recorded. Proximate values of each fish were determined (section 2.17) from collected and preserved samples following the methods of AOAC (1980).

3.2.12 Statistical analysis

The triploid yields; inter female difference in response to particular treatments of optimal pressure, heat and cold shocks; differences in gonad weight and GSI between diploid and triploid fish at subsequent age groups; comparative performance of growth and growth related parameters, biochemical and proximate values, serum calcium and endocrine hormone levels between and among ploidy groups such as diploid control (2n Cont.), triploid pressure shock (3n P.S.), triploid heat shock (3n H.S.) and triploid cold shock (3n C.S.); all these structured experimental designs were analysed using a one way ANOVA (Statgraphics Computer Package, Version 3.0, Statistical Graphic Corporation, STSC, Inc.). This included: significant results $P<0.05$ were taken as rejection of the null hypothesis - significant differences between groups. Significant results were further analysed using Scheffe's Multiple Range Tests in order to determine ranking and significant differences between treatment means. These results are displayed as superscripts against each respective value.

Simple linear regression analysis was used to check for relationships between gonad weight and body weight among diploid and triploid groups of succeeding age class. A Chi-square test of goodness-of-fit was used to determine whether frequencies in occurrence of sexes (both male amd female) differed from the expected 1:1 ratio in each ploidy group. All these statistical analyses were also carried out using Statgraphics (Version 3.0).

3.3 RESULTS

3.3.1 Triploidy induction using pressure, heat and cold shocks

The results of the present study for the identification of treatment optima in inducing triploidy by exposing newly fertilised eggs of *O. niloticus* to altered intensities, durations and timings of application of hydrostatic pressure, heat and cold shocks are described. The following treatments are close to the individual agent optima to produce all triploidy: Pressure: 8,000 p.s.i., 2 mins. duration applied 9 mins. after fertilisation (a.f.); Heat: 41 °C, 3.5 mins. duration applied 5 mins. a.f.; Cold: 9 °C, 30 mins. duration applied 7 mins. a.f.

3.3.1.1 Optimisation of hydrostatic pressure shock treatments:

It is convenient to begin by first explaining the results of those experiments conducted using high hydrostatic pressure. This is appropriate since, because no changes in temperature were involved in their execution, they can be considered as being the most informative about the normal timing of the events of the second meiotic division at the temperature used (28°C). The full list of experiments conducted and results obtained is given in Table 3.3.1.1. The averaged results, showing the effects on triploid yields of altering pressure level and timings of application of 2 mins. duration shocks, are presented in a more convenient format in Fig. 3.3.1.1. All levels of 2 mins. duration pressure shock were most effective to induce triploidy when given in the period between 8 - 10 mins. after fertilisation (a.f.) at 28 °C; earlier (5 - 7 mins. a.f.) administrations of 7000 - 8000 p.s.i. shocks did not produce any viable triploid progeny except very few deformed survivors. Later (11 - 12 mins.) applications of shock resulted in lowered triploid rates at all pressure levels. The optimum timing identified

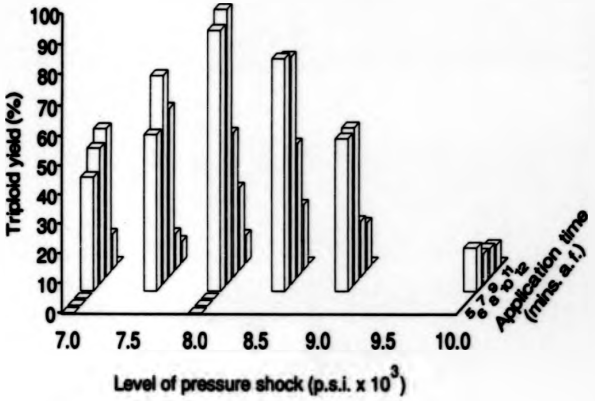
Table 3.3.1.1. The effects of different intensities, durations and timings of application of pressure shock on triploid rates and triploid yields in *O. niloticus*.

Pressure Level (p.s.i.)	Time (mins. a.f.)	Shock dur[a] (mins.)	Survival rate to hatch(%RC')	Triploid rate (%±SE)	Triploid yield (%±SE)
7000	5[c]	2	0	0	0
	6[c]	2	11.4± 1.8	0	0
	7[c]	2	26.7± 4.2	0	0
	8[b]	2	92.8± 4.7	41.7±13.0	38.7±13.6
	9[b]	2	83.2± 4.5	55.6± 4.5	46.2± 5.0
	10[c]	2	91.7± 4.3	55.0± 3.5	50.4± 5.6
	11[c]	2	94.4± 0.6	12.6± 1.0	11.9± 1.1
	12[c]	2	75.9± 9.1	0	0
7500	8[b]	2	78.7± 7.4	67.6±11.4	53.2±13.2
	9[b]	2	86.9± 2.2	80.3± 3.3	69.8± 4.6
	10[c]	2	81.5± 7.0	70.0± 7.0	57.0± 0.9
	11[c]	2	80.2± 6.0	15.1± 0.6	12.1± 0.4
	12[c]	2	70.6±16.5	5.0± 3.5	3.5± 2.5
8000	5[c]	2	0	0	0
	6[c]	2	0	0	0
	7[c]	2	0	0	0
	8[c]	2	87.5± 5.5	100	87.5± 5.5
	9[b]	1	79.6± 8.8	0	0
	9[b]	1.5	77.2±12.0	61.3± 5.0	47.3± 9.4
	9[c]	2	91.8± 2.1	100	91.8± 2.1
	9[b]	2.5	64.4±16.9	100	64.4±16.9
	9[b]	3	43.8±10.6	100	43.8±10.6
	9[b]	4	14.0±10.6	66.7±27.2	9.3±10.0
	10[b]	2	78.8± 3.5	62.2± 5.0	49.0± 5.9
	11[b]	2	85.6± 3.5	32.6± 5.7	27.9± 5.7
	12[b]	2	73.6± 8.9	4.1± 3.4	3.0± 2.4
8500	8[b]	2	78.0±10.3	100	78.0±10.3
	9[b]	2	76.2± 3.6	100	76.2± 3.6
	10[c]	2	72.6± 6.9	62.5± 8.8	45.4± 7.0
	11[c]	2	73.3± 6.7	30.0± 3.2	22.0± 4.6
	12[c]	2	55.2± 1.1	0	0
9000	8[b]	2	52.0±14.6	100	52.0±14.6
	9[b]	2	53.2± 8.8	100	53.2± 8.8
	10[c]	2	57.3± 2.1	32.8± 3.3	18.8± 3.9
	11[c]	2	63.3± 5.0	25.0± 5.8	15.8± 5.0
	12[c]	2	50.0± 2.0	0	0
10000	8[c]	2	34.0± 6.5	43.3± 9.8	14.7± 6.0
	9[c]	2	37.8± 1.4	27.2± 5.5	10.3± 2.5
	10[c]	2	55.1± 7.8	17.5± 5.3	9.6± 1.6
	11[c]	2	75.6± 2.3	5.5± 3.9	4.2± 3.0
	12[c]	2	57.4±13.5	0	0

Experiments were conducted using [a]2, [b]3 or [c]4 different females.
RC' Relative to control after adjustment of latter to 100%.
Mean survival rate in controls was 75.8±2.0%(N = 23).

Figure 3.3.1.1

The effect different intensities and times of application of 2 mins. duration pressure on triploid yield in *O. niloticus*.


Triploid yield (%)
100
90
80
70
60
50
40
30
20
10
0
7.0
7.5
8.0
8.5
9.0
9.5
10.0
Level of pressure shock (p.s.i. x 10³)
6
7
8
9
10
11
12
Application time (mins. a.f.)

for exposing fertilised eggs for 2 mins. duration to induce high triploid rates and high triploid yields at most effective pressure levels (8000 - 8500 p.s.i.) was 9 mins. a.f. Lower levels of shock (7000 - 7500 p.s.i.), administered at 8 - 9 mins. a.f. resulted in generally lowered triploid yields because triploid rates were lower. At higher pressure (9000 - 10,000 p.s.i.) triploid yields once again reduced but, in this case, it was a consequence of both lowered rates and fewer animals surviving the treatments. An intermediate pressure shock of 8000 p.s.i. administered 9 mins. a.f. for 2 mins. duration was found to be close to the treatment optima (Table 3.3.1.1 and Fig. 3.3.1.1) in inducing maximum triploid rates (100%) at maximum triploid yields (91.8±2.1 %). Shock durations shorter than 2 mins. were less effective because they produced fewer triploids and longer shocks also gave poor triploid yields because they allowed fewer survivors.

3.3.1.2 Optimisation of heat shock treatments:

The results of the heat shock experiments are presented in detail in Table 3.3.1.2. Averaged results from all fish showing the effects on yields of alterations in temperature and duration of heat shocks applied 5 mins. a.f. are presented graphically in Fig. 3.3.1.2. Heat shocks of 37, 38 and 39 °C applied at 5 mins. a.f., even those of long duration, were largely without effect on survival rates but were ineffective in inducing triploidy. Increased temperatures of heat shock (40 - 42 °C) were effective in inducing triploidy but, as the temperature of heat was increased, the maximally effective duration of heat shock appeared to become shorter. Thus, at 40 °C the most effective duration of shock, administered 5 mins. a.f., was between 5 - 6 mins. whilst at 41 and 42 °C this duration fell to 3 - 4 and 3 mins. respectively. At the highest temperature, the width of the window in which triploid yields could be produced at high levels also, apparently, became reduced. Heat shocks of 3.5 mins.

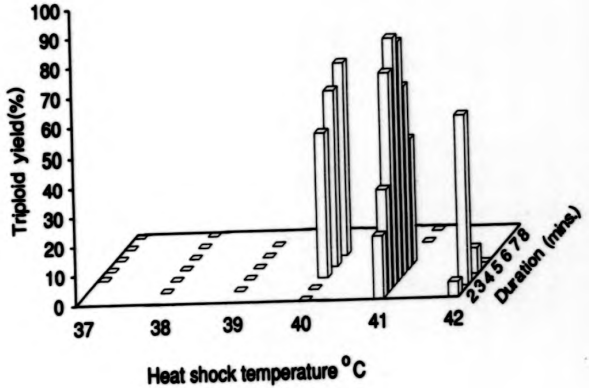
Table 3.3.1.2. The effects of different intensities, durations and timings of application of heat shock on triploid rates and triploid yields in *O. niloticus*.

Temp. of shock (±0.2°C)	Time (mins. a.f.)	Shock dur[a] (mins.)	Survival rate to hatch(%RC*)	Triploid rate (%±SE)	Tripoid yield (%±SE)
	5[a]	4	99.3±0.3	0	0
	5[a]	5	94.1±1.8	0	0
37	5[a]	6	91.5±3.2	0	0
	5[a]	7	90.8±0.2	0	0
	5[a]	8	91.3±1.0	0	0
	5[a]	10	90.2±1.7	0	0
	5[a]	3	93.4±3.8	0	0
	5[a]	4	94.3±2.7	0	0
38	5[a]	5	98.9±0.4	0	0
	5[a]	6	93.0±1.7	0	0
	5[a]	7	81.8±4.3	0	0
	5[a]	8	87.7±2.6	0	0
	5[a]	3	82.8±1.2	0	0
	5[a]	4	91.6±2.0	0	0
39	5[a]	5	91.7±1.4	0	0
	5[a]	6	89.6±1.7	0	0
	5[a]	7	93.1±3.3	0	0
	5[a]	2	94.9±2.0	0	0
	5[a]	3	83.9±7.6	0	0
40	5[a]	4	84.5±1.7	57.8±1.5	48.8±2.3
	5[a]	5	83.8±2.2	70.8±2.9	59.3±4.0
	5[a]	6	81.8±4.2	79.1±2.9	64.7±3.0
	5[b]	2	36.4±0.4	58.9±3.9	21.4±1.2
	5[b]	2.5	49.0±3.1	72.2±3.2	35.4±0.7
	5[b]	3	72.3±2.2	100	72.3±2.2
	2[b]	3.5	60.0±5.0	54.2±5.8	32.5±5.6
	3[b]	3.5	60.7±5.8	67.1±7.2	40.7±7.4
	4[b]	3.5	69.6±6.4	92.7±2.8	64.5±4.2
41	5[b]	3.5	82.1±5.3	100	82.1±5.3
	6[b]	3.5	85.7±4.7	72.6±6.8	62.2±9.2
	7[b]	3.5	83.4±1.8	23.1±5.3	19.3±4.2
	5[b]	4	78.8±0.7	100	78.8±0.7
	5[b]	4.5	61.9±5.5	100	61.9±5.5
	5[b]	5	43.6±5.4	96.7±2.7	42.2±4.9
	5[b]	7	0	0	0
	5[a]	2	38.6±3.4	13.0±2.1	5.0±1.3
42	5[a]	3	57.4±0.4	100	57.4±0.4
	5[a]	4	9.8±2.1	90.0±3.5	8.8±2.7
	5[a]	5	0	0	0

Experiments were conducted using [a]2 or [b]3 different females.
RC* Relative to controls after adjustment of the latter to 100%.
Mean survival rate in controls was 84.4±1.9%(N = 16).

Figure 3.3.1.2

The effect of different temperatures and durations of heat shock on triploid yield in *O. niloticus* when administered 5 mins. after fertilisation.


Triploid yield(%)
100
90
80
70
60
50
40
30
20
10
0
37
38
39
40
41
42
2 3 4 5 6 7 8
Duration (mins)
Heat shock temperature °C

duration at 41±0.2 °C were the most effective in inducing maximum triploid rates (100%) and maximum triploid yields (82.1±5.3 %) when delivered 5 mins. a.f. (Table 3.3.1.2 and Fig. 3.3.1.2). Earlier timings (2 - 3 min. a.f.) of administration of this shock caused generally lowered survival and triploid rates and therefore lowered triploid yields. Some of the animals which survived early application of heat shock were aneuploid (hyperdiploid or hypotriploid) and this presumably explains the greater lethality of both early administered heat and pressure shocks. Later (6 - 7 mins. a.f.) applications of heat shock (41 °C) at 3.5 mins. duration sharply declined triploid rates but did not affect survival rates.

3.3.1.3 Optimisation of cold shock treatments:

The results of those trials performed using cold shock treatments are detailed in Table 3.3.1.3 and the averaged results of the effects on triploid yield of alteration in temperature and duration of cold shocks administered at 7 mins. a.f. are presented in Fig. 3.3.1.3. Triploids were produced by all temperatures of cold shock in the range 7 - 15 °C, the most effective duration of shock being 20 - 40 mins. Any duration above this level resulted in lower survival rates which consequently lowered triploid yields. No triploids were produced when eggs were submitted to a cold shock of 11 °C for 60 mins. at timings in the range of 5 -11 mins. a.f. Earlier (5 -6 mins. a.f.) application of 11 °C shock resulted in lowered triploid rates and triploid yields. Triploid rate declined sharply close to 0% with later (8 - 11 mins. a.f.) application of cold shock. At 11 °C the most effective time of administration of 40 mins. duration shock was 7 mins. a.f. to induce maximum 97.0±2.7% triploidy. Triploid rates (100%) and triploid yields (85.7±9.7%) were apparently highest after the application of 30 mins. duration at 9°C shock administered at 7 mins. a.f.(Table 3.3.1.3 and Fig. 3.3.1.3). Although triploids were induced by the application of lower temperature

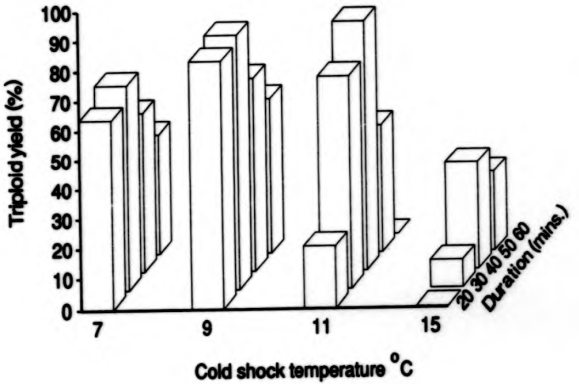
Table 3.3.1.3. The effects of different intensities, durations and timings of application of cold shock on triploid rates and triploid yields in *O. niloticus*.

Temp. of shock (±0.2°C)	Time (mins. a.f.)	Shock dur[a] (mins.)	Survival rate to hatch(%RC')	Triploid rate (%±SE)	Triploid yield (%±SE)
7	7[a]	20	87.7± 8.5	72.5±1.7	63.6±4.6
	7[a]	30	69.2± 5.8	100	69.2±5.8
	7[a]	40	76.1±15.8	100	53.8±15.840.
	7[a]	50	67.5± 8.0	60.0±7.0	40.5±0.1
9	7[b]	20	96.0± 1.3	86.5±2.8	83.0±3.6
	7[b]	30	85.7± 9.7	100	85.7±9.7
	7[b]	40	65.1± 4.4	100	65.1±4.4
	7[b]	50	52.1± 5.8	100	52.1±5.8
11	5[a]	30	91.8± 0.1	25.0±3.5	22.9±4.0
	5[a]	60	3.4± 0.3	0	0
	6[a]	30	98.5± 0.4	68.3±1.1	67.3±0.9
	6[a]	40	73.6± 5.0	72.3±4.0	53.2±6.6
	7[b]	20	87.5± 5.3	23.3±3.6	20.4±2.7
	7[b]	30	85.0± 0.3	84.2±1.8	71.5±1.2
	7[b]	40	86.5± 4.8	97.0±2.7	83.9±4.0
	7[b]	50	51.5± 3.1	83.3±3.6	42.9±4.1
	7[b]	60	0.5± 0.3	0	0
	8[a]	20	91.6± 5.2	0	0
	8[a]	30	90.2± 0.9	31.3±4.4	28.2±4.3
	8[a]	40	78.1±12.0	55.0±5.5	42.9±9.3
	9[a]	30	17.3± 2.3	18.3±1.1	3.2±0.2
	9[a]	60	3.6± 2.5	0	0
	11[a]	30	45.3± 7.4	0	0
	11[a]	60	17.1±11.2	0	0
15	7[a]	20	82.3± 3.9	0	0
	7[a]	30	56.4± 7.5	16.3±2.6	9.2±2.7
	7[a]	40	61.6± 1.2	58.3±5.9	35.9±2.0
	7[a]	50	41.8± 6.6	63.3±2.3	26.5±5.2

Experiments were conducted using [a]2 or [b]3 different females.
RC' Relative to control after adjustment of the latter to 100%.
Mean survival rate in controls was 78.4±2.3%(N = 13).

Figure 3.3.1.3

The effect of different temperature and durations of cold shock on triploid yield in *O. niloticus*.


Triploid yield (%)
100
90
80
70
60
50
40
30
20
10
0
7
9
11
15
Cold shock temperature °C
20 30 40 50 60
Duration (mins.)

shocks (7 °C), lowered tripoid yields were associated with increased numbers of deformed animals at this temperature. Higher temperature cold shocks (15 °C) were generally less effective in inducing triploidy.

3.3.1.4 Comparative trials for triploid yields by optimal pressure, heat and cold shocks:

It is arguably more important to outline the general nature of response to different agents and to contrive, as it was attempted to do, to reduce them to a common understanding which might be of use in the identification of preferred options. Therefore, it is more important to identify any inter-individual difference in response to particular treatments, and this was the rationale behind the second part of this study where the apparently preferred agent optima were applied in a directly comparable manner, to the eggs of 8 different females (Table 3.3.1.4). As expected, all animals karyologically analysed (10 per treatment group) were made triploid by the optimised pressure (8000 p.s.i., 2 mins. duration applied 9 mins. a.f.), heat (41±0.2 °C, 3.5 mins. duration applied 5 mins. a.f.) and cold (9±0.2 °C, 30 mins. duration applied 7 mins. a.f.) shocks and therefore the difference between agents related to variability in the survival rates. Mean triploid yields following pressure shock (84.8±3.4%RC, N=8) was significantly greater ($P<0.05$) than those seen after cold shocking (37.2±11.6%RC, N=8). The cold shock survivals showed a lot of inter female difference despite the uniformly high control and pressure shocks survivals. Although, in most instances, individual yield values were greater after exposure to pressure rather than after exposure to heat treatments, mean yields following heat shock (70.3±5.5%RC, N=8) were not significantly different ($P>0.05$) from pressure shocks.

Table 3.3.1.4. The control survival rates and comparative triploid yields following the exposure of eggs from 8 different females to optimal* pressure, heat and cold shocks.

Fish no	Control survival rate (%)	Triploid yield (% RC')		
		Pressure	Heat	Cold
1	94.0	91.5	68.6	90.6
2	91.5	67.0	67.1	13.6
3	76.3	85.8	90.9	3.9
4	93.3	74.2	69.4	20.7
5	85.8	83.9	75.7	81.6
6	82.0	93.3	86.3	61.6
7	91.7	84.0	69.0	21.6
8	76.4	98.9	35.5	4.0
Mean±SE	86.4±2.4[a]	84.8±3.4[a]	70.3±5.5[ab]	37.2±11.6[b]

*The shocks used were as follows:
Pressure: 8000 lb p.s.i., 2 mins duration applied 9 mins. a.f.
Heat : 41 °C, 3.5 mins. duration applied 5 mins. a.f.
Cold : 9 °C, 30 mins. duration applied 7 mins. a.f.

RC'Relative to control after adjustment to survival rates in the later to 100%. Triploid rates following all treatments were 100%(N=10).

3.3.2 Effects of triploidy on sexual maturation and reproduction

3.3.2.1 Sexual development of diploids and triploids in successive age groups:

Table 3.3.2.1A presents the data of mean standard length, body weight, gonad weight and GSI for female and male *O. niloticus* belonging to successive age groups. This experiment was not designed as a growth study and the results from the small monthly samples do not give a good estimate of the true differences in diploid and triploid performance. These measurement were incidental to calculation of ovary weight and GSI. A more detailed results of a study on comparative performances of growth, biochemical composition and endocrine profiles of diploids and three groups of triploids (pressure, heat and cold shocked) are presented in Section 3.3.3 of this text.

In this study it was observed that diploid female ovaries were 20 - 50 times heavier than ovaries of their triploid sibs, they were large and packed with numerous developing oocytes from 4 months of age to the end of the growing period (10 months) . In contrast, triploid ovaries were very thin, string-like and sometime short and plump and containing a few small oocytes (Fig. 3.3.2.1a). From 5 to 10 months of age highly significant differences ($P<0.001$) in ovary weight and GSI were found between triploid and diploid females (Table 3.3.2.1A). A comparison between triploid and diploid female GSI from 4 to 10 months of age is shown graphically in Fig. 3.3.2.1e. The mean ovary weight and body weight of females in both ploidy levels were positively correlated during the course of study period and the values of the correlation co-efficient(R) for linear regression equation were significant in most cases. During the course of sexual maturation diploid females showed

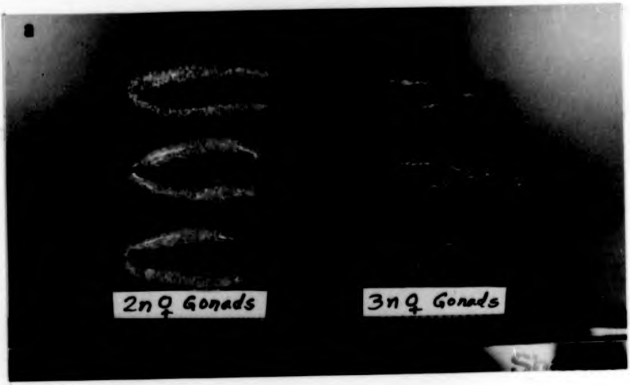
Table 3.3.2.1A. Mean standard length, body weight, gonad weight and gonadosomatic index (GSI) for female and male tripoid and diploid *Oreochromis niloticus* in successive age groups (4,5,6,7,8, and 10 months). All values are mean±SE based on 5 fish in each category. Significance level of ANOVA for comparisons between 2n and 3n of each sex at every age are given against 3n sex ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$).

	Ploidy/Sex	Age (months)					
		4	5	6	7	8	10
Length (cm)	2n Female	11.10±0.20	13.10±0.50	13.20± 0.90	15.90± 0.50	17.50± 0.40	18.00± 0.04
	3n Female	10.10±0.20	11.70±0.80	13.60± 0.30	14.50± 0.40	17.70± 0.40	19.00± 0.30
	2n Male	12.20±0.40	14.50±0.10	15.60± 0.80	17.80± 0.40	18.20± 0.40	18.80± 0.30
	3n Male	11.60±0.40	13.40±0.30	14.20± 0.60	16.40± 0.60	17.60± 0.50	18.60± 0.20
Weight (g)	2n Female	56.80±3.00	88.20±7.90	95.90±19.10	164.20±18.60	212.90±14.70	227.00± 9.50
	3n Female	44.20±2.80*	73.90±0.30	123.90± 6.00	147.90±14.10	238.60±10.50	257.50± 5.30
	2n Male	71.60±7.70	119.70±5.00	193.40±10.70	231.40±17.30	252.70±11.30	305.90±20.80
	3n Male	70.80±4.70	100.00±6.60	118.70± 9.90**	210.30±22.90	219.30± 9.90	280.70±17.90
Gonad weight (g)	2n Female	0.50±1.60	1.60±0.20	1.70± 0.20	3.30± 0.40	4.60± 0.50	5.50± 0.90
	3n Female	0.01±0.00	0.03±0.01***	0.08± 0.01***	0.10± 0.01***	0.20± 0.02***	0.30± 0.02**
	2n Male	0.40±0.10	1.70±0.60	2.30± 0.30	2.70± 0.30	4.50± 1.10	5.50± 0.60
	3n Male	0.40±0.10	1.40±0.20	1.90± 0.60	3.30± 0.30	3.80± 0.40	4.80± 0.40
GSI	2n Female	0.82±0.44	1.80±0.30	1.80± 0.20	2.00± 0.30	2.20± 0.30	2.40± 0.40
	3n Female	0.02±0.00	0.04±0.10***	0.06± 0.10***	0.07± 0.01***	0.08± 0.01***	0.10± 0.01**
	2n Male	0.55±0.09	1.40±0.38	1.20± 0.20	1.20± 0.20	1.80± 0.40	1.80± 0.20
	3n Male	0.56±0.13	1.40±0.19	1.60± 0.30	1.60± 0.20	1.70± 0.20	1.70± 0.10

Figure 3.3.2.1

(a) Showing diploid and triploid ovaries at 10 months old *O. niloticus*.

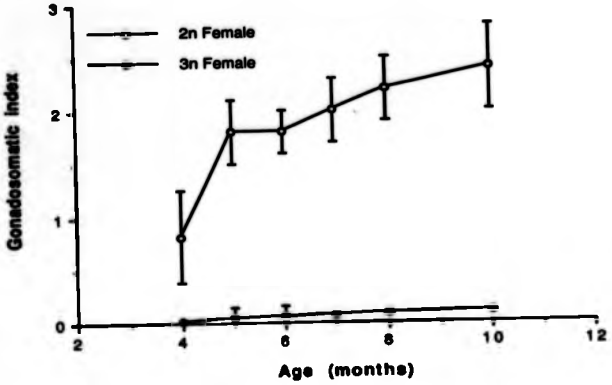
(b) Showing diploid and triploid testes at 10 months old *O. niloticus*.


2n ♀ Gonads
3n ♀ Gonads


2n ♂ Gonads
3n ♂ Gonads

Figure 3.3.2.1e

A comparison of diploid and triploid female GSI in *O. niloticus* from 4 to 10 months of age.


2n Female
3n Female
Gonadosomatic index
3
2
1
0
2
4
6
8
10
12
Age (months)

normal development of the urogenital papilla whereas the papillae of the triploid females were very poorly developed (Fig. 3.3.2.1c).

Diploid male testes were soft, elongate, milky coloured and full of spermatozoa. Triploid testes were more or less similar size in overall, but were thin and flat and watery coloured milt (Fig. 3.3.2.1b). No significant differences ($P>0.05$) in testes weight and GSI were found between triploid and diploid males. A positive correlation was also found between mean testes weight and body weight for males of both ploidy levels and "R" values were also significant. Morphologically, triploid males showed similar development of secondary sexual characters having the prominent urogenital papilla and shiny body colour of normal mature diploid males (Fig. 3.3.2.1d).

In addition to the above study, the serum concentration of calcium (index of vitellogenin), 17β-oestradiol and 11β-hydroxytestosterone levels were measured in both sexes of diploid and triploid sibs belonging to three successive age groups (6, 7 and 8 months). The data is given in Table 3.3.2.1B. In diploid females the mean serum calcium level was significantly greater ($P<0.05$) than triploid females at 6 and 7 months and the values were relatively higher in month 8 but not significantly different. The same trend in 17β-oestradiol level was evident among the females of the two ploidy groups and the values at 6 and 7 months of age were significantly greater ($P<0.05$) in the diploid females although by month 8 the levels were the same. In the earlier (6 and 7) months a direct correlation was observed between the mean values of GSI, serum calcium concentration and 17β-oestradiol in both diploids and triploids females, which had again disappeared by 8 months. No significant

Figure 3.3.2.1

(c) Showing urogenital papilla in diploid and triploid female *O. niloticus* at sexual maturation.

(d) Showing urogenital papilla in diploid and triploid male *O. niloticus* at sexual maturation.


2N
3N


2N
3N

Table 3.3.2.1B. Mean serum calcium (index of vitellogenin), 17β-oestradiol and 11β-hydroxytestosterone levels including GSI for female and male diploid and triploid *Oreochromis niloticus* in successive age groups (6, 7 and 8 months). All values are means±SE based on 5 fish in each category except those shown in parentheses. Significance Differences of ANOVA and Scheffe,s Multiple Range Tests for comparisons between 2n and 3n of each sex at every age are given against 3n sex ($^{*}P<0.05$; $^{***}P<0.001$).

Parameters	Ploidy/Sex	Age (months)		
		6	7	8
GSI	2n Female	2.79±0.38	1.54±0.47***	1.73±0.36***
	3n Female	0.06±0.01***	0.07±0.01***	0.10±0.01***
	2n Male	1.03±0.39	1.19±0.25	1.37±0.37
	3n Male	2.61±0.25	1.87±0.23	0.97±0.20
Serum calcium (mg/100 ml)	2n Female	25.05±0.07	22.21±1.97	17.70±0.69
	3n Female	12.15±0.20*	13.10±0.17*	15.91±0.13
	2n Male	13.44±0.44	13.30±0.15	15.98±0.53
	3n Male	13.35±0.57	13.18±0.15	15.18±0.32
17β-oestradiol (ng ml^{-1})	2n Female	13.82±4.02	19.72±5.68	10.32±3.84
	3n Female	3.59±0.61*	4.96±1.67*	11.11±6.17
	2n Male	N.D.	N.D.	N.D.
	3n Male	N.D.	N.D.	N.D.
11β-hydroxy testosterone (ng ml^{-1})	2n Female	N.D.	N.D.	N.D.
	3n Female	N.D.	N.D.	N.D.
	2n Male	4.37±0.98(4)	3.65±0.65	4.66±0.72(4)
	3n Male	5.49±0.16	2.23±0.28(4)	6.77±3.41(3)

N.D. = Not determined

variation was found between diploid and triploid males for mean values of serum calcium and 11β- hydroxytestosterone levels in this experiment.

3.3.2.2 Gonad histology of diploids and triploids in successive age groups:

Histological sections of diploid ovaries at 4 and 5 months of age had a strongly basophilic cytoplasm and lightly stained round nuclei in the developing primary and previtellogenic oocytes. At 6 - 8 months of age ovaries contained oogonia and many maturing previtellogenic and vitellogenic oocytes with irregular nuclei and vacuolated cytoplasm which is associated with endogenous and exogenous yolk formation (Fig. 3.3.2.2a). The follicles of vitellogenic oocytes consist of a transparent theca, granulosa and zona radiata. At later stages (8 - 10 months), atresia of some of the vitellogenic oocytes was also occurring.

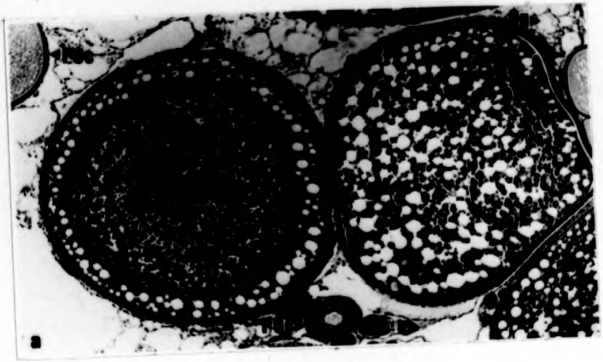
Histological sections of triploid ovaries showed they contained mainly oogonia and a few small primary or previtellogenic oocytes (Fig. 3.3.2.2b). In early stages (4 - 7 months) most of the cells were of similar size with very few undergoing divisions. In later stages (8 - 10 months) some of the primary or young previtellogenic oocytes started to show retarded development. In the present study, no secondary or vitellogenic oocytes were found in triploid ovaries even at peak of the maturation time for diploids of the same sib group.

Histological sections of diploid testes of successive age groups contained highly distinct cysts surrounded by the basal lamina at all stages of development. There were spermatogonia, primary or secondary spermatocytes, spermatids and a large number of spermatozoa in testicular sections after maturation (Fig. 3.3.2.2c). In contrast, triploid testes

Figure 3.3.2.2 Histological sections of *O. niloticus* ovaries at 10 months of age (H & E, 77x).

(a) Diploid ovary showing oogonia (Og), primary or previtellogenic oocytes (POc) and vitellogenic oocytes (VOc).

(b) Triploid ovary containing mostly oogonia (Og) and few small primary or previtellogenic oocytes (POc).


Pbs
a

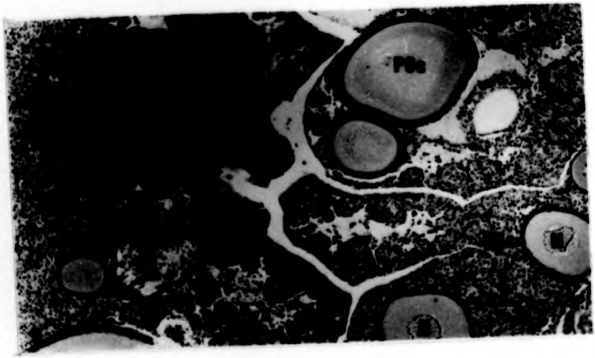

Pbs

Figure 3.3.2.2 Histological sections of *O. niloticus* testes at 10 months of age (H & E, 304x).

(c) Diploid testis showing spermatogonia (SpO), spermatocytes (SpC), spermatids (SpT) and a huge number of spermatozoa (SpZ).

(d) Triploid testis containing cysts mostly with spermatogonia (SpO), spermatocytes (SpC), some spermatids (SpT) and a few spermatozoa (SpZ) in seminal fluid (SF).

contained mostly cysts with spermatogonia and spermatocytes. In testicular sections of some triploids, germ cells were under active divisions and had developed into spermatids and spermatozoa: others were blocked during the course of spermatogenesis possibly at the first meiotic division. In the later stages (8 - 10 months) most of the cysts began to fuse with degenerating sperm cells resulting in nearly empty tubules having lightly stained seminal fluid consisting of a few spermatozoa with large nuclei (Fig. 3.3.2.2d). There were very few spermatozoa in milt stripped from triploid males many of which showed abnormal morphology and motility.

3.3.2.3 Reproductive performance of triploid males crossed with diploid females:

The data for mean survival rate of embryos at four developmental stages viz. morula (MOR), pigmentation (PIG), hatch (HAT) and yolk sac resorption (YSR) derived from 10 different crosses between triploid males and diploid females is plotted in Fig. 3.3.2.3. The results revealed that triploid sperms were mostly unable to fertilize normal eggs. In a few crosses, where fertilization (2.7 - 44%) occurred, the hatched larvae were found to be deformed and died before YSR. Karyotypic analysis revealed that hatched embryos from such crosses were all aneuploid (between $2n = 44$ and $3n = 66$). In contrast, in all crosses between diploid males and females about 98 - 100% of the eggs were fertilized. Embryo survival to YSR ranged from 62.5 to 93.7%, karyologically all fry were normal diploids ($2n = 44$) and there were no deformed fry.

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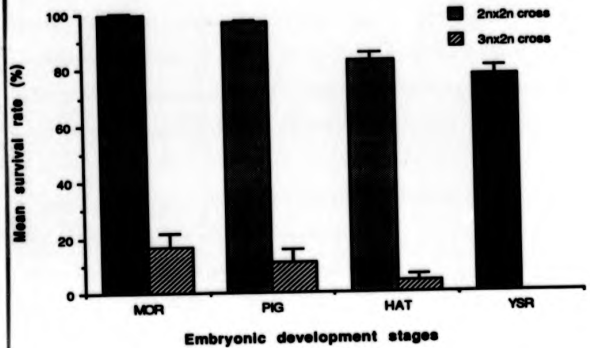
Figure 3.3.2.3

Histograms showing mean survival rate of embryos at four development stages viz. morula (MOR), pigmemtation (PIG), hatch (HAT) and yolk sac resorption (YSR). Embryos derived from 10 crosses between 2n female x 2n male and 2n female x 3n male.


2nx2n cross
3nx2n cross
Mean survival rate (%)
100
80
60
40
20
0
MOR
PIG
HAT
YSR
Embryonic development stages

3.3.3 Comparative growth, biochemical composition and endocrine profiles of diploids and pressure, heat and cold shocked triploids

3.3.3.1 Performance of growth and relative parameters:

The comparative data for growth experiments conducted in 4 experimental tanks showing the results in detail by weight and length of mixed sex diploid control (2n Cont.), triploid pressure (3n P.S.), triploid heat (3n H.S.) and triploid cold (3n C.S.) *O. niloticus* belonging to successive age groups (10, 15, 20, 25, 30, 35 and 40 weeks) are given in Table 3.3.3.1A and 3.3.3.1B. The pooled mean data for weight and length of 4 ploidy groups from all tanks are presented in Fig. 3.3.3.1A and 3.3.3.1B respectively.

At stocking (10 weeks of age), the mean weight of fish belonging to 2n Cont., 3n P.S., 3n H.S. and 3n C.S. groups was not significantly different between and among ploidy groups in each tank, although there were differences between tanks. In most of the tanks until 25 weeks the growth increment of all the replicated groups was rapid but the 2n Cont. and 3n C.S. groups were faster. From 30 weeks, just after sexual maturation the 2n Cont. group showed comparatively slower growth rate whereas faster growth in weight and length for 3n H.S. and 3n C.S. was observed until 40 weeks. During the whole on-growing period 3n P.S. group grew slower compared to other groups. At 40 weeks the maximum growth in mean weight (269.3±15.4SE g) and length (17.8±0.3SE cm) was attained by 3n C.S. and 3n H.S. groups respectively (Fig. 3.3.3.1A and 3.3.3.1B). However from 25 weeks onward until 40 weeks both weight and length comparisons for 2n Cont., 3n P.S., 3n H.S. and 3n C.S. revealed no significant differences ($P>0.05$) between and among ploidy groups (Table 3.3.3.1A and 3.3.3.1B).

Table 3.3.3.1A. Data for growth in weight(g) for mixed sex diploid control (2n Cont.), triploid pressure shock (3n P.S.), triploid heat shock (3n H.S.) and triploid cold shock (3n C.S.) *O. niloticus* belonging to successive age groups (10, 15, 20, 25, 30, 35 and 40 weeks). All values are mean based on equal number of fish (N = 12) per replication in each tank. Significant differnces of ANOVA and Scheffe,s Multiple Range Tests for comparisons between and among the ploidy groups in each tank are shown by superscripts against each value.

Tanks	Ploidy	10 wks	15 wks	20 wks	25 wks	30 wks	35 wks	40 wks
No. 1	2n Cont.	2.08^{a}	7.26^{a}	24.0^{a}	75.2^{a}	114.6^{a}	183.9^{a}	219.7^{a}
	3n P.S.	2.11^{a}	11.91^{a}	18.8^{a}	78.4^{a}	105.1^{a}	166.1^{a}	213.2^{a}
	3n H.S.	2.03^{a}	11.13^{a}	21.7^{a}	85.8^{a}	105.7^{a}	174.7^{a}	267.9^{a}
	3n C.S.	3.02^{a}	12.45^{a}	25.2^{a}	97.2^{a}	105.3^{a}	183.4^{a}	248.5^{a}
No. 2	2n Cont.	1.85^{a}	7.06^{a}	16.5^{a}	72.9^{a}	127.4^{a}	220.8^{a}	302.5^{a}
	3n P.S.	1.42^{a}	4.41^{a}	16.5^{a}	63.9^{a}	76.8^{a}	172.9^{a}	242.0^{a}
	3n H.S.	1.23^{a}	4.72^{a}	23.8^{a}	63.0^{a}	118.4^{a}	185.6^{a}	281.8^{a}
	3n C.S.	2.33^{a}	6.15^{a}	28.4^{a}	71.4^{a}	92.2^{a}	164.4^{a}	228.4^{a}
No. 3	2n Cont.	1.79^{a}	8.13^{a}	17.5^{a}	62.6^{a}	87.7^{a}	141.8^{a}	214.9^{a}
	3n P.S.	0.97^{a}	4.64^{a}	14.0^{a}	48.5^{a}	92.8^{a}	140.4^{a}	209.7^{a}
	3n H.S.	0.99^{a}	7.63^{a}	14.4^{a}	60.1^{a}	100.1^{a}	160.5^{a}	206.6^{a}
	3n C.S.	1.68^{a}	11.22^{a}	12.6^{a}	51.6^{a}	118.2^{a}	189.1^{a}	279.0^{a}
No. 4	2n Cont.	1.42^{a}	6.10^{a}	14.5^{b}	49.3^{a}	128.9^{a}	218.1^{a}	299.4^{a}
	3n P.S.	0.60^{a}	1.60^{b}	6.3^{a}	33.9^{a}	104.1^{a}	177.3^{a}	245.3^{a}
	3n H.S.	0.73^{a}	2.44^{b}	6.2^{a}	41.1^{a}	111.8^{a}	182.2^{a}	284.8^{a}
	3n C.S.	0.79^{a}	6.89^{a}	11.4^{ab}	45.9^{a}	125.9^{a}	227.9^{a}	321.6^{a}

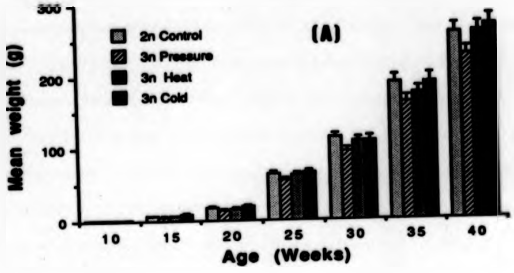
Table 3.3.3.1B. Data for growth in length(cm) for mixed sex diploid control (2n Cont.), triploid pressure shock (3n P.S.), triploid heat shock (3n H.S.) and triploid cold shock (3n C.S.) *O. niloticus* belonging to successive age groups (10, 15, 20, 25, 30, 35 and 40 weeks). All values are mean based on 12 fish per replication in each tank. Significant differences of ANOVA and Scheffe,s Multiple Range Tests for comparisons between and among ploidy groups in each tank are shown by superscripts.

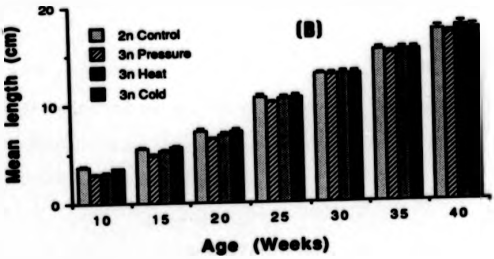
Tanks	Ploidy	10 wks	15 wks	20 wks	25 wks	30 wks	35 wks	40 wks
No. 1	2n Cont.	3.67^{a}	5.63^{a}	7.91^{a}	11.2^{a}	13.4^{a}	15.5^{a}	17.0^{a}
	3n P.S.	3.50^{a}	6.00^{a}	6.95^{a}	11.3^{a}	13.2^{a}	15.3^{a}	16.8^{a}
	3n H.S.	3.52^{a}	6.46^{a}	7.81^{a}	11.9^{ab}	13.1^{a}	15.4^{a}	18.0^{a}
	3n C.S.	4.02^{a}	6.41^{a}	8.02^{a}	14.4^{b}	12.9^{a}	15.5^{a}	17.4^{a}
No. 2	2n Cont.	3.49^{a}	5.56^{a}	7.18^{a}	10.9^{a}	13.2^{a}	15.9^{a}	18.0^{a}
	3n P.S.	3.18^{a}	4.89^{a}	7.10^{a}	10.6^{a}	12.1^{a}	15.1^{a}	17.2^{a}
	3n H.S.	2.97^{a}	4.84^{a}	7.91^{a}	10.5^{a}	13.2^{a}	15.7^{a}	18.1^{a}
	3n C.S.	3.60^{a}	4.91^{a}	8.13^{a}	10.9^{a}	11.9^{a}	14.6^{a}	16.7^{a}
No. 3	2n Cont.	3.40^{a}	5.66^{a}	7.29^{a}	10.8^{a}	12.4^{a}	14.3^{a}	16.4^{a}
	3n P.S.	2.67^{b}	4.69^{a}	6.55^{a}	9.7^{a}	12.3^{a}	14.4^{a}	17.4^{a}
	3n H.S.	2.94^{ab}	5.56^{a}	6.90^{a}	10.4^{a}	13.0^{a}	14.9^{a}	16.6^{a}
	3n C.S.	3.50^{a}	5.80^{a}	6.38^{a}	9.6^{a}	13.0^{a}	15.4^{a}	17.4^{a}
No. 4	2n Cont.	3.27^{a}	5.26^{a}	6.85^{a}	10.1^{a}	13.2^{a}	15.9^{a}	18.2^{a}
	3n P.S.	2.52^{b}	3.61^{b}	5.22^{a}	9.0^{a}	13.9^{a}	15.3^{a}	17.2^{a}
	3n H.S.	2.67^{b}	4.00^{b}	5.20^{ab}	9.3^{a}	13.0^{a}	15.5^{a}	18.5^{a}
	3n C.S.	2.80^{ab}	5.16^{a}	6.28^{b}	9.5^{a}	14.1^{a}	15.6^{a}	18.5^{a}

Figure 3.3.3.1

(A) Mean body weight (g) of 2n control, 3n pressure, 3n heat and 3n cold shock groups of *O. niloticus* belonging to successive age groups (10, 15, 20, 25, 30, 35 and 40 weeks).

(B) Mean body length (cm) of 2n control, 3n pressure, 3n heat and 3n cold shock groups of *O. niloticus* belonging to successive age groups (10, 15, 20, 25, 30, 35 and 40 weeks.


(A)
2n Control
3n Pressure
3n Heat
3n Cold
Mean weight (g)
300
200
100
0
10
15
20
25
30
35
40
Age (Weeks)


(B)
2n Control
3n Pressure
3n Heat
3n Cold
Mean length (cm)
20
10
0
10
15
20
25
30
35
40
Age (Weeks)

Data of all other relative growth parameters viz. mean gutted weight, percentage dress-out, gut weight, condition factor, liver weight and hepatosomatic index together with mean weight and length for female and male in the four groups at the end of the experiment (45 weeks) are presented in Table 3.3.3.1C. At 45 weeks of age all triploid females (3n P.S., 3n H.S. and 3n C.S.) were heavier but not significantly different ($P>0.05$) than 2n Cont. females. The mean gutted fish weight in 3n C.S. females (232.3±20.9 g) was significantly greater ($P<0.05$) than 2n Cont. females (170.3±8.5 g) but not from the other two triploid groups (3n P.S. and 3n H.S.) which were themselves not significantly different from the 2n Cont. Both 3n H.S. and 3n C.S. females showed significantly higher ($P<0.05$) percentage dress-out over 2n Cont. which was not significantly different ($P>0.05$) from the 3n P.S. females, and the mean values were not significantly different ($P>0.05$) among the triploid groups. There were significant differences in gut weight ($P<0.05$) and K factor ($P<0.01$) between 3n C.S. and 2n Cont. females. Statistically no significant differences ($P>0.05$) were observed for liver weight and HSI among the females of treatment groups.

In males no significant differences ($P>0.05$) were found in mean length, percentage dress-out, gut weight, HSI and K factor among the groups. Mean weight, gutted fish weight and liver weight were significantly less in 3n P.S. males compared to 3n C.S. which was not different from 3n H.S. and 2n Cont.

3.3.3.2 Biochemical and proximate composition:

Table 3.3.3.2 represents data for mean crude protein, crude lipid, moisture and ash determined from muscle samples collected from females and males from the four groups at 45 weeks of age. Mean crude protein concentration in females did not differ significantly

Table 3.3.3.1C. Mean standard length, body weight, gonad weight, GSI, gutted fish weight, percentage dressout, liver weight, HSI, gut weight and condition factor for female and male diploid control (2n Cont.), triploid pressure shock (3n P.S.), triploid heat shock (3n H.S.), triploid cold shock (3n C.S.) *O. niloticus* estimated at 45 weeks of age. All values are mean calculated on 15 fish of either sex of each ploidy group.Values in parentheses are ±SE. Significant differnces of ANOVA and Scheffe,s Multiple Range Tests for comparisons of all growth parameters between and among groups of each sex are shown by superscripts against each value.

Growth parameters	Female				Male			
	2n Cont.	3n P.S.	3n H.S.	3n C.S.	2n Cont.	3n P.S.	3n H.S.	3n C.S.
Length(cm)	17.1^{a} (±0.3)	16.9^{a} (±0.4)	17.8^{a} (±0.4)	17.9^{a} (±0.5)	19.8^{a} (±0.4)	18.8^{a} (±0.4)	20.2^{a} (±0.4)	19.4^{a} (±0.3)
Weight(g)	191.0^{a} (±9.2)	193.9^{a} (±12.0)	219.1^{a} (±12.3)	252.1^{a} (±22.3)	332.6^{ab} (±16.7)	279.3^{b} (±15.5)	339.0^{ab} (±13.9)	364.7^{a} (±20.0)
Gonad weight(g)	4.7^{a} (±1.2)	0.19^{b} (±0.02)	0.21^{b} (±0.02)	0.22^{b} (±0.02)	1.8^{a} (±0.3)	1.6^{a} (±0.2)	1.3^{a} (±0.1)	2.0^{a} (±0.3)
GSI	2.46^{a} (±0.29)	0.10^{b} (±0.01)	0.10^{b} (±0.01)	0.09^{b} (±0.01)	0.54^{a} (±0.09)	0.57^{a} (±0.06)	0.38^{a} (±0.03)	0.56^{a} (±0.07)
Gutted fish weight(g)	170.3^{b} (±8.5)	177.1^{ab} (±11.5)	202.2^{ab} (±11.4)	232.3^{a} (±20.9)	306.5^{ab} (±15.6)	257.5^{b} (±14.5)	316.8^{ab} (±12.9)	332.6^{a} (±17.2)
Percentage dress-out	89.2^{b} (±0.6)	91.3^{ab} (±0.5)	92.3^{a} (±0.4)	92.1^{a} (±0.4)	92.1^{a} (±0.2)	92.2^{a} (±0.3)	93.4^{a} (±0.2)	92.9^{a} (±1.4)
Liver wieght(g)	4.3^{a} (±0.2)	3.7^{a} (±0.3)	5.1^{a} (±0.4)	5.1^{a} (±0.5)	6.6^{a} (±0.5)	4.9^{b} (±0.4)	6.7^{a} (±0.4)	7.3^{a} (±0.6)
HSI	2.30^{b} (±0.25)	1.91^{ab} (±0.08)	2.30^{b} (±0.09)	2.00^{ab} (±0.10)	1.98^{a} (±0.12)	1.75^{a} (±0.08)	2.00^{a} (±0.08)	2.00^{a} (±0.10)
Gut weight(g)	6.1^{b} (±0.5)	8.0^{ab} (±0.4)	8.1^{ab} (±0.6)	9.5^{a} (±1.0)	9.2^{a} (±0.9)	8.6^{a} (±0.6)	8.6^{a} (±0.6)	9.9^{a} (±0.7)
K factor	3.4a^{b} (±0.1)	3.6^{a} (±0.1)	3.6^{a} (±0.07)	3.8^{a} (±0.1)	3.9^{b} (±0.1)	3.9^{b} (±0.2)	3.8^{b} (±0.1)	4.5^{a} (±0.1)

Table 3.3.3.2. Data for biochemical composition in female and male diploid control (2n Cont.), triploid pressure shock (3n P.S.), triploid heat shock (3n H.S.) and triploid cold shock (3n C.S.) *O. niloticus* determined at 45 weeks of age. All values are mean±SE based on equal number (N = 5) of fish in either sex. Significant differences of ANOVA and Scheffe,s Multiple Range Tests for comparison of crude protein, crude lipid, moisture and ash together with GSI between and among the groups of each sex are shown by superscripts against each value.

Ploidy	Sex	No. of Fish	GSI	Crude protein (%)	Crude lipid (%)	Moisture (%)	Ash (%)	NFE (By difference)
2n Cont.	Female	5	2.69±0.42^{a}	84.9±0.5^{a}	7.2±0.5^{a}	78.0±0.2^{a}	5.6±0.1^{a}	2.2
3n P.S.	Female	5	0.12±0.03^{b}	83.9±0.5^{a}	9.6±0.3^{a}	78.1±0.2^{a}	5.6±0.1^{a}	0.9
3n H.S.	Female	5	0.12±0.01^{b}	84.2±1.4^{a}	8.7±0.4^{a}	77.4±0.4^{a}	5.2±0.06^{a}	1.8
3n C.S.	Female	5	0.11±0.03^{b}	85.3±0.6^{a}	7.3±0.8^{a}	77.7±0.2^{a}	5.7±0.1^{a}	1.7
2n Cont.	Male	5	0.33±0.04^{a}	86.6±0.4^{a}	7.2±0.5^{a}	79.3±0.2^{a}	5.3±0.1^{b}	0.8
3n P.S.	Male	5	0.35±0.09^{a}	86.8±1.1^{a}	6.3±1.1^{a}	79.6±0.5^{a}	5.7±0.1^{a}	1.1
3n H.S.	Male	5	0.37±0.04^{a}	85.9±0.5^{a}	6.8±0.5^{a}	79.0±0.2^{a}	5.6±0.1^{ab}	1.7
3n C.S.	Male	5	0.37±0.06^{a}	85.8±0.7^{a}	6.9±0.3^{a}	78.1±0.5^{a}	5.6±0.07^{ab}	1.6

between and among ploidy groups. In all triploid females (around 8.5±0.5 %, N=5) crude lipid deposition in muscle was higher but not significantly different from 2n Cont. group (7.2±0.5 %, N=5). Similarly no significant differences ($P>0.05$) were found for moisture and ash in females of all ploidy groups.

In males the only difference observed was that of ash, which was significantly lower ($P<0.05$) in 2n Cont. compared to 3n P.S. males.

3.3.3.3 Gonadal condition and sex ratio:

A comparison of gonad weight and GSI for female and male in the four groups at 45 weeks of age is shown in Table 3.3.3.1C. In 2n Cont. females ovaries were large and full of developing eggs, while in all triploid females ovaries were mostly thin string-like and surrounded by mainly oogonia and small oocytes. This study revealed highly significant differences ($P<0.001$) of ovary weight and GSI between 2n Cont. and the three triploid groups (3n P.S., 3n H.S. and 3n C.S.).

In 2n Cont. males the size of testes did not differ morphologically in comparison to 3n P.S., 3n H.S. and 3n C.S. males. Therefore, no significant differences ($P>0.05$) in testis weight and GSI were found between and among the males of all ploidy groups.

The sex ratio of experimental fish of four ploidy groups (N = 48 fish in each group) was determined at the end of the experiment (45 weeks of age). The observed frequency of male and female sexes in 2n Cont. and three triploid groups was not significantly different from the expected 1:1 ratio (Table 3.3.3.3).

Table 3.3.3.3. Observed frequency of male and female sexes of diploid control (2n Cont.), triploid pressure shock (3n P.S.), triploid heat shock (3n H.S.) and triploid cold shock (3n C.S.) *O. niloticus* at 45 weeks of age. Numbers in parentheses are expected numbers of both sexes.

Ploidy	Fish no.	Sexes(no.) Male	Female	Chi-square (Sig. level)
2n Cont.	48	25(24)	23(24)	0.84 N.S.
3n P.S.	48	23(24)	25(24)	0.84 N.S.
3n H.S.	48	25(24)	23(24)	0.84 N.S.
3n C.S.	48	26(24)	22(24)	0.68 N.S.

N.S.= Not significant

3.3.3.4 Serum calcium concentration (index of vitellogenin):

Table 3.3.3.4 gives the mean serum calcium levels together with GSI for females and males of the four groups. At 45 weeks of age the serum calcium concentration in 2n Cont. females was greater ($P<0.001$) compared to those of females belonging to 3n female groups. There was also a correlation between GSI and serum calcium level of diploid and triploid females at the 45 weeks of age.

In males significant variations ($P<0.05$) in serum calcium concentration were also observed between the 2n Cont., 3n P.S. and 3n C.S. groups but not the 3n H.S., where the values of this parameter remained close to basal levels (around 15.0 mg/100 ml).

3.3.3.5. Sex steroid hormone levels:

At 45 weeks of age sex steroid hormones (testosterone, 11-ketotestosterone and 17β-oestradiol) were determined in female and male sexes of four ploidy groups and data are presented in Table 3.3.3.5. There were significantly higher ($P<0.001$) mean testosterone levels in 2n Cont. females (39.07±7.09 ng/ml) than in the three triploid groups (around 16.78±2.38 ng/ml). 17β-oestradiol levels in 3n P.S. females (3.82±1.10 ng/ml) were significantly lower ($P<0.01$) than 2n Cont. females (12.54±2.30 ng/ml). In 3n H.S. and 3n C.S. groups of females, the values of this steroid hormone were also below the limits of 2n Cont. females but not significant different ($P>0.05$) from either 2n Cont. or 3n P.S. groups.

Mean testosterone and 11-ketotestosterone in all triploid male groups assayed at the same age (45 weeks) of females were comparatively lower than 2n Cont. males and revealed no significant differences among ploidy groups.

Table 3.3.3.4. Data for serum vitellogenin in female and male diploid control (2n Cont.), triploid pressure shock (3n P.S.),triploid heat shock (3n H.S.) and triploid cold (3n C.S.) *O. niloticus* belonging to 45 weeks of age. All values are mean±SE based on equal number (N=7) of fish in each category. Significant differences of ANOVA and Scheffe,s Multiple Range Tests for comparisons of GSI and serum calcium level between and among ploidy groups of either sex are shown by superscripts against each value.

Ploidy	Sex	Fish no.	GSI	Serum total total calcium (mg/100 ml)
2n Cont.	Female	7	2.50±0.26^{a}	34.28±2.3^{a}
3n P.S.	Female	7	0.10±0.01^{b}	15.84±0.2^{b}
3n H.S.	Female	7	0.09±0.01^{b}	14.86±0.2^{b}
3n C.S.	Female	7	0.09±0.02^{b}	13.73±0.6^{b}
2n Cont.	Male	7	0.42±0.04^{a}	16.46±0.5^{a}
3n P.S.	Male	7	0.48±0.07^{a}	14.39±0.3^{b}
3n H.S.	Male	7	0.61±0.07^{a}	14.77±0.6^{ab}
3n C.S.	Male	7	0.51±0.09^{a}	14.09±0.4^{b}

Table 3.3.3.5. Data for serum steroid hormone levels in female and male diploid control (2n Cont.), triploid pressure shock (3n P.S.), triploid heat shock (3n H.S.) and triploid cold shock (3n C.S.) *Oreochromis niloticus* at 45 weeks of age. All values are mean±SE estimated from an equal number (N = 10) of fish in each category. Significant differences of ANOVA and Scheffe,s Multiple Range Tests for comparison of testosterone, 11-ketotestosterone and 17-β oestradiol together with GSI between and among ploidy groups of either sex are shown by superscripts against each value.

Ploidy	Sex	No. of Fish	GSI	Testosterone (ng ml^{-1})	11-ketotestosterone (ng ml^{-1})	17β-oestradiol (ng ml^{-1})
2n Cont.	Female	10	2.50±0.23^{a}	39.07±7.09^{a}	N.D.	12.54±2.30^{a}
3n P.S.	Female	10	0.12±0.02^{b}	16.63±2.68^{b}	N.D.	3.82±1.10^{b}
3n H.S.	Female	10	0.13±0.04^{b}	15.47±1.99^{b}	N.D.	9.11±1.58^{ab}
3n C.S.	Female	10	0.11±0.02^{b}	18.24±2.47^{b}	N.D.	6.33±1.25^{ab}
2n Cont.	Male	10	0.52±0.12^{a}	82.07±17.25^{a}	37.45±12.55^{a}	N.D.
3n P.S.	Male	10	0.49±0.08^{a}	42.75± 5.80^{a}	13.05± 5.00^{a}	N.D.
3n H.S.	Male	10	0.35±0.04^{a}	75.59±28.31^{a}	31.70±10.00^{a}	N.D.
3n C.S.	Male	10	0.60±0.10^{a}	44.04±11.57^{a}	18.92± 7.28^{a}	N.D.

N.D.= not determined

3.4 DISCUSSION

3.4.1 Optimisation of treatment parameters for triploidy induction in newly fertilised eggs

The results of these trials performed to induce triploidy using high hydrostatic pressure are consistent with the hypothesis that the meiotic spindle apparatus of developing eggs is susceptible to disturbance during a very narrow window of time, approximately two minutes, between 8 - 10 minutes after fertilisation at 28 °C. Later treatments, though allowing survival, are progressively less effective in inducing triploidy because the 2nd meiotic division has already been completed. Earlier administration of shocks resulted in no triploidy and the survival of a few deformed embryos, possibly because of improper timing and deleterious effects of the treatments resulted in the production of inviable aneuploids. Pressure shock intensities above and below the optimum resulted in incomplete retention of the 2nd polar body producing aneuploidy and deformity and killed more embryos reducing triploid rates and yields. Longer and shorter pressure shock durations either side of the optimum also sharply reduced the triploid yields due to a drastic effect on survival rate. Therefore, treatment optima can be considered as residing on a plateau, the slopes of which it is necessary to "climb" or to avoid "sliding down" in order to have the most consistent effect. A closer inspection of the triploid yield variance figures suggests that, as might be expected if such a scenario were true, variability was greatest in those trials which, in retrospect, might be considered sub-optimal i.e. shocks given too early or too late or at undesirable durations or intensities.

From the above discussion it is clear that pressure shock treatments can be deleterious if the conditions are sub-optimal. This finding is supported by the studies of Chourrout (1984) and Elahi (1990). Chourrout (1984) observed a high frequency of abnormal aneuploid embryos caused by incomplete retention of the 2nd polar body at the lower intensities of pressure shocks during the induction of triploidy in rainbow trout. In a detailed experiment on the effect of sub-optimal pressure shock in inducing triploidy in *O.niloticus* Elahi (1990) found that sub-optimal shock durations, time after fertilisation and low shock intensity produced higher rates of aneuploidy, lower survival and fewer triploids.

Varadaraj and Pandian (1986) showed that a 7500 p.s.i. pressure shock administered for 2 - 15 mins. duration at 2.5 mins. a.f. was most effective in inducing 100% triploidy in *O. mossambicus*. This situation indicates that pressure shock treatments might be species specific. Their timing for application of pressure shocks on newly fertilised eggs are not consistent with the recent study, because the present findings in *O. niloticus* revealed that any earlier application of pressure shocks before 8 mins. a.f. resulted in no or few deformed survivors (aneuploids) and no triploids. On the other hand, 7500 p.s.i. shock intensity was effective but not reliable in inducing 100% triploidy in *O. niloticus*. The other report on ploidy manipulation using hydrostatic pressure in *O. niloticus* is the study on tetraploid induction by Myers (1986). He reported that pressures in the region of 6500 - 7500 p.s.i., that is lower than those shown to be maximally effective in the present study as inducing triploidy, were effective, when used in combination with low temperatures (7.5 °C), in suppressing the first mitotic division. Although it will be shown later that this temperature of cold shock is effective, when of sufficient duration to induce triploidy by itself, the duration of 7.5 °C shock used by Myers (1986) is shorter than that reported by this text to

be maximally effective. This may mean that the slight difference in pressure ranges reported by these two studies are the result of pressure and cold shock acting synergistically in the study of Myers (1986) rather than indicating that the optima for interference with the meiotic event are intrinsically different from those necessary to disturb mitotic division processes.

From the present results it appears that the most effective heat shocks must be administered earlier in the post fertilisation period than must the most effective pressure shocks. It could be concluded that as increased heat has the effect of advancing the metabolic rate of all biological processes, the timing of effective administration of heat shock is therefore earlier. This has the effect of reducing the width of the effective window for interference compared to the correspondingly effective pressure shock. A picture therefore emerges, in the case of heat shock exposure, that there are conflicting consequences of treatment. Heat shocks tend to accelerate eggs through a window in which it is necessary for them to be retained in order to have maximal effect. Essentially similar results and a similar hypothesis to explain them have been advanced to explain the effects of heat shock generation of triploids in Atlantic salmon (Johnstone, 1989).

As with pressure shocks, sub-optimal conditions for heat shock has a profound effect on embryonic survival, triploid yields, deformity and aneuploidy. Similar results of sub-optimal heat shock intensities and durations on the survival, triploid rates and triploid yields of *O. niloticus* were seen by Elahi (1990) working on the same stock of fish.

These results of optimal heat shock treatment are essentially similar to those of Chourrout and Itskovich (1983) and Penman et al. (1987a) but are slightly different from those of Don and Avtalion (1986, 1988a) and Varadaraj and Pandian (1988, 1990). Don and Avtalion (1986) advocated the use of a lower temperature (39.5 - 40.5 °C) shock of 3.4 - 4 mins. duration at 3 - 3.5 mins. a.f. in the same species. A heat shocking of 42 °C for a period of 3 mins. at 2.5 mins. a.f. was applied by Varadaraj and Pandian (1988) in *O. mossambicus*. Shock intensities above and below 41 °C were found to be sub-optimal in the present trials and in the study of a previous worker (Elahi, 1990) and early timings performed poorly in inducing triploidy in *O. niloticus*.

The results of optimisation of cold shock treatments in the present study are comprehensive and entirely different than previous work on cold shock induction of triploidy in *Oreochromis spp.*(Valenti 1975; Don and Avtalion 1988a). Previous workers used only one temperature (11 °C), one duration (60 mins.) and various timings (0.5 - 20 mins. a.f.) of application of shock. These shock parameters gave poor results in the present trials. No viable triploidy could be induced because of the greater lethality and deleterious effect of cold shock on embryonic survival of this particular shock intensity (11 °C) and duration (60 mins.). This study further revealed that colder shock temperature (7 °C) relative to the optimum induces all triploidy with an increased number of deformed embryos at reduced yields.

On the basis of the present findings a conclusion could be drawn that cold shocks, as they cause a slowing down of the rate of the meiotic division events, have necessarily to be applied for a relatively longer period than is necessary for either heat or pressure but must

be initiated at approximately similar timing as pressure treatments, in order to be effective. Closer inspection of the results suggest that the timing of initiation of optimum cold shock should be slightly earlier than an equivalent pressure shock. This may, if true, indicate that these agents have different lag times before exerting their effects.

Compreshensive though the above trials are, it is yet possible to argue that far more extensive trials are required to properly identify the nature of response to the various agents under discussion. Thus, it could be concluded that for all agents, not only is duration but also the intensity and timing of exposure of importance in maximising yields. At elevated intensities of treatment the present results show that shorter durations of exposure and alternative timings are indicated. It appears likely, therefore, that rather than there being one treatment optimum for a given agent, there are more likely to be families of treatment optima comprising exposures of different intensities and duration each having a particular timing for best effect but, perhaps, not differing substantially in yield. Thus, in the context of the present study it might be argued, although the experiment was not performed, that a pressure shock of short duration (say 1 min.) of 10000 p.s.i. administered at 8 mins. a.f., might have resulted in similar triploid yields as the apparently preferred 8000 p.s.i. shock of 2 mins. duration that are reported. Similarly a lower intensity of heat shock (e.g. 40 °C of 6 mins. duration) might need to be administered earlier for maximal effect (e.g. 4 mins. a.f.) than was actually performed.

Finally the extent of inter-individual response and variation in triploid yields was carefully monitored in this study with comparative trials following the exposure of eggs from different females to optimal pressure, heat and cold shocks, where pressure had been shown

to have certain advantages over heat and cold in inducing triploidy. The cold shock in this study which was effective as an optimum for eggs of one female was observed to be less ideal, even lethal, for those of another, therefore resulting in high inter female variations. Heat shock showed lower inter-individual variation than was found for cold, but on average were not as great as pressure in optimizing triploid yields. A similar situation following temperature shock for the induction of triploids, in the use of heat to produce triploid Atlantic salmon has been reported where, because of extreme variability in response to the same heat shock treatment of eggs from different females, pressure shock was the preferred method of triploidization (Benfey and Sutterlin, 1984a; Benfey et al., 1988; Johnstone, 1989). The reason might be pressure shock is less damaging to the embryos than temperature shock (Thorgaard, 1983). Other workers also came to more or less the same conclusion during comparison of heat and hydrostatic pressure shocks to induce triploidy in steelhead trout (Guixiong et al., 1989) and salamanders (Gillespie and Armstrong, 1979). These species differences in response to temperature may be the consequences of specific temperature adaptations or may relate to temperature equilibration differences. Lincoln (1989) reported that pressure shock has some advantages over heat as a means of inducing triploidy in rainbow trout, which gives lower egg and fry mortalities. He stated that the major advantage is the ease with which large batches of eggs may be processed because of the way in which pressure treatment uniformly affects all of the eggs: this is not always easily achieved using heat because large volumes of eggs impair heat transfer which may lead to reduced triploid rates.

It might be presumed that the preferred method of ploidy manipulation in any species would be that which was most specific in it's action on the spindle apparatus as well as that which

conformed most closely to having a "square wave" effect at exposure. Since heat shocks, by accelerating development, have the effect of narrowing the window of opportunity for triploidization making timing of application of shock more critical and inter-individual response to cold shocks is apparently greater, therefore, the present results demonstrate that pressure treatments come closest to this ideal for the generation of *O. niloticus* triploids.

3.4.2 Gonadal development, sexual maturation and reproduction in diploid and triploid individuals

The real aim of inducing triploidy in the present fish was to develop a sterile population in an attempt to prevent precocious sexual maturation and fertility. As early maturation processes in normal tilapia, particularly, females often have profound and, ultimately, limiting effects on growth resulting from the prolific breeding and overpopulation. Therefore, the effects of triploidy on gonadal development and sexual maturation were examined in female and male *O. niloticus* belonging to successive age groups as one of the important part of this performance study. The present results of external, cytological and histological appearance of triploid ovaries from 4 to 10 months of age revealed continuous suppression of functional oocyte development possibly as a result of severe disruption of the normal process of gametogenesis during early meiotic division. This might have some direct correlation with the absence of serum vitellogenin and minimum level of sex steroid hormones in triploid females, which will be discussed later in this part of the text. As a result the triploid ovaries remained undeveloped and smaller at all stages of maturation compared to those of diploid sibs. Similar conditions have been observed by several workers in the ovaries of triploid plaice and plaice x flounder hybrids (Purdom, 1972; Lincoln, 1981b), channel catfish (Wolters et al., 1982; Chrisman et al., 1983), loach (Suzuki

et al. 1985), European catfish (Krasnai and Marian, 1986), African cat fish (Richter et al., 1987); rainbow trout (Thorgaard and Gall, 1979, Lincoln and Scott, 1984; Nakamura et al., 1987); Thai silver carp (Na-Nakorn and Legrand , 1991) and *O. mossambicus* (Pandian and Varadaraj 1988). Vitellogenic oocytes have been observed in triploid ovaries of *O. aureus* by Penman (1989). This phenomenon might therefore be species or even strain specific. Such eggs seem likely to be aneuploid, as was described for the eggs of triploid amphibians (Fankhauser and Humphrey, 1950; Kawamura, 1951).

The present study further revealed that the size of triploid testes (4 to 10 months of age) of *O niloticus* did not differ significantly in comparison to their diploid sibs. However, during the course of spermatogenesis, there was histological evidence that there was mass atresia and degeneration of germ cells. The few active divisions of spermatocytes could result in aneuploid spermatozoa, ultimately leading to functional sterility of the testes. These observations are consistent with the results reported on triploid testis development in stickleback (Swarup, 1957), plaice and plaice x flounder hybrids (Lincoln, 1981a), channel catfish (Wolters et al., 1982), Atlantic salmon (Benfey and Sutterlin, 1984b) and African catfish (Richter et al., 1987).

The reproductive sterility of triploid *O. niloticus* males observed in this study by crossing some of them with diploid females resulting in all aneuploid progeny is essentially similar to that of plaice, *Pleuronectes platessa* (Lincoln, 1981a) and blue tilapia, *O. aureus* (Penman et al., 1987a).

3.4.3 Performance of growth, biochemical composition and endocrine profiles of diploids and pressure, heat and cold shocked triploids

Some workers (Cassani and Caton, 1986; Lincoln and Bye, 1987) have already shown that triploids do not grow well in competition to diploids when they are grown together, but these findings are not consistent with the results of the present study. No significant differences were found in the growth rate among mixed sex ploidy groups from 10 to 40 weeks of on-growing period (see- section 3.3.3.1) suggesting no detrimental or negative effect resulted from such combined rearing. Similar observations have been reported for mixed sex experiments on three *Oreochromis* spp. (*O. niloticus, O. aureus* and *O. mossambicus*) by Penman et al. (1987a). They also observed that triploids induced by heat shock were significantly smaller and mortalities were higher in comparison to diploids during embryonic and larval development. Other workers on Atlantic salmon (Benfey and Sutterlin, 1984b, Benfey et al., 1989a); loach (Suzuki et al., 1985a); coho salmon (Johnson et al., 1986) and African catfish (Richter et al., 1987; Henken et al., 1987) did not find any difference in growth rate between diploids and triploids. In common carp, juvenile triploids did not grow faster than diploid ones (Gervai et al., 1980). One exception in the early growth (7 - 13 weeks) performance study of cold shocked induced triploidy in European catfish (*Silurus glanis* L.) was reported by Krasznai and Marian (1986), who found significantly higher values of growth rate in triploids than those of diploid counterparts. Similarly Valenti (1975) observed polyploid *T. aurea* grew faster during their 14 weeks of age than diploids. During the spawning period of diploid counterparts, adult triploid fish gained more weight and became heavier in plaice x flounder hybrids (Purdom, 1976; Lincoln, 1981c) ; rainbow trout (Thorgaard and Gall, 1979) and channel catfish (Wolters et al., 1982).

At the completion of the growth experiment of the present study, when the sex of individual fish was fully differentiated, it was found that the mean weight and length for females of 3n H.S. and 3n C.S. growers were comparatively higher as well as percentage dress-out than the diploid group at 45 weeks of age. Lincoln (1981c) in a growth study of female triploid plaice x flounder hybrids concluded that triploids appeared to have no great advantage over diploids except for fillet weight which was found to be significantly greater in these fish. A likely explanation of the relatively superior growth and percentage dress-out of such triploid *O. niloticus* females is that they expend less or no energy on ovarian development compared to diploid females at the stage of their normal sexual maturation and become fatter and bulkier (Lincoln, 1981c; Wolters et al., 1982; Henken et al., 1987). On the other hand, matured female diploids show reduced somatic growth because energy is diverted to developing ovaries during the period of spawning (Lincoln, 1981c). Among the triploid groups, 3n C.S. females showed significantly higher K factor and gut weight over diploid control group. It was observed in this study that all triploid females accumulated excessive fat bodies around viscera, therefore attained higher gut weight compared to the 2n Cont. females. In mature rainbow trout Lincoln and Scott (1984) showed similar significant differences in dress-out percentage, K factor and gut weight between diploid and triploid females. In *O. niloticus* (Penman et al. 1987a), triploid females showed significantly higher dress-out percentages rather than K factor values compared to diploids.

The absence of significant differences in most of the above growth parameters measured between diploid and triploid male groups is common to other studies such as triploid *Oreochromis* spp. (Penman et al. 1987a), rainbow trout (Lincoln and Scott, 1984) and coho

salmon (Johnson et al., 1986). The only significant difference was for K value in the 3n C.S. males which in this study showed superiority in growth rate for length over the three other ploidy groups just after the normal period of sexual maturation of diploids. This is similar to the results in rainbow trout triploid males as reported by Lincoln and Scott (1984).

In the context of the present study, although the overall growth performance of triploid groups is not significantly better than the diploids, it can be concluded that triploid tilapia might show better performance under natural pond conditions as opposed to laboratory based tanks. The reason is that in natural condition diploid tilapias will spawn frequently and the grow-out ponds will became heavily overcrowded with young fish. The fry will compete each other for available space and food, therefore, ultimately they will be stunted in growth. But this situation will not happen in case of ponds stocked with completely sterile triploid populations.

The biochemical composition analysis of muscle in the present experiment was conducted to see if there might be some variations in proximate values between the ploidy groups due to onset of the maturation cycle of normal diploids. There is evidence that there are changes in histology, GSI and the biochemical composition of various body tissues such as muscle, ovary and hepatopancreas (protein, fat and carbohydrate) as the reproductive state of marine invertebrates changes (Penaflorida and Millamena, 1990). It seems therefore that the level of protein and lipid concentration in particular may be influenced as maturity progresses in fish. Castille and Lawrence (1989) observed changes in carbohydrate, lipid and protein content in gonads and digestive glands in *Penaeus* spp. during their gonadal maturation. In

O. niloticus no definite trend in variation of muscle protein was correlated with the change of sexual maturation status among ploidy groups of either sex in this study. Similarly in diploid and triploid *Carassius auratus*, the muscle protein composition did not differ clearly between the two groups (Sezaki et al., 1983). The muscle lipid concentration in the present experimental fish did not show any significant difference between diploid and triploid groups but the level in 3n females was consistently higher than all other groups. This might correlate with the fat deposition observed around the internal organs in these fish. A similar phenomenon was observed in triploid female rainbow trout (Thorgaard and Gall, 1979). In contrast, there was relatively little lipid deposition in diploid female *O. niloticus*, similar to the situation observed in rainbow trout by Lincoln and Scott (1984). They suggested that female diploids utilize the lipid deposits for vitellogenesis during the breeding season, which does not occur for obvious reason in triploid fish.

Triploids did not show any variation in muscle moisture content values in any of the groups. On the other hand, variability in crude ash content between triploid pressure and diploid males can not readily be explained. Sezaki et al. (1983) reported that no differences in muscle moisture nor ash content between diploid and triploid *C. auratus* were distinguishable.

In conclusion it is clearly apparent in this study that no significant pattern of variation in biochemical composition except ash, were found among the ploidy groups of either sex, unlike other gonadal and growth parameters.

The failure of the ovaries to develop in the 3n female group could be a response to the

mechanical difficulties likely to occur at the 1st meiosis (Lincoln, 1981a). It is however clear from the serum calcium (index of vitellogenin) and other endocrine hormone levels observed in these ploidy groups that 3n females lacked the correct hormone balance to support ovarian growth. Tyler, Sumpter and Bromage (1987a, 1987b) explained that egg yolk is derived from a precursor lipophosphoprotein-calcium complex called vitellogenin which is synthesised by liver, the vitellogenin is released into the blood and finally sequestered by the oocytes by means of a receptor-mediated endocytotic process. The yolk precursor is believed to be synthesised during the vitellogenesis phase of ovarian growth under the influence of oestrogenic control (Pan et al., 1969). The serum calcium concentration estimated as an index of vitellogenin secretion in all triploid female groups in the present study suggested extreme suppression of ovarian growth and showed no detectable concentration of vitellogenin, which could be considered as "basal level". The absence of serum calcium concentration above the basal level in the blood serum of triploid *O. niloticus* females indicated that there was no exogenous vitellogenesis such as occurring during the normal maturation of their diploid sibs. During this time the reduced liver weight, expressed as HSI, in most of the triploid female groups was influenced by the low level of 17β-oestradiol hormone which would normally support vitellogenin production. Therefore, triploid ovaries could not show any sign of oocyte maturation due to the lack of yolk deposition (Jonson et al., 1986; Benfey et al., 1989b; Ng and Idler, 1983). No significant variation was observed in serum calcium concentration between 3n males groups which were close to basal levels. The significantly higher levels of serum calcium concentration seen in the diploid male group compared to the 3n males in the present study can not readily be explained. One possibility is that there might be a correlation of serum

calcium concentration with relatively higher androgen hormone levels in the serum of matured diploid males.

The reduced level of sex steroid hormones (testosterone and 17β -oestradiol) in the serum of all triploid female groups further correlates with the suppression of oocyte development of these fish compared to the diploids. As a major site for sex steroid production is the follicular cell layers surrounding the oocytes (Hoar, 1965; Nagahama, 1983), in triploid females this is much reduced, whereas the oocytes in diploid ovary had an extensive mass of steroidogenic tissues (Johnson et al., 1986) to support the production of sex steroid hormones during the period of sexual maturation.

There is evidence that in oviparous vertebrates vitellogenin synthesis is regulated by the ovarian hormone 17 β-oestradiol (Tata, 1978; Wallace, 1978). In fish, several workers have demonstrated that 17β-oestradiol elevates vitellogenin in the serum of females during their reproductive cycle (Elliot et al., 1979; Whitehead et al., 1978; Scott et al., 1980; Bromage et al.,1982a,1982b). Eckstein and Katz (1971); Colombo et al.(1972) and Lambert and Pot (1975) observed that androgens are the major biosynthetic products of *in vitro* incubation of ovarian tissues. Since testosterone is converted to 17β-oestradiol by the aromotase enzyme several authors have concluded that testosterone acts as a precursor in oestrogen synthesis (Campbell and Idler, 1976; Scott et al., 1982).

The present data show a direct correlation between the level of serum calcium concentration (index of vitellogenin) and sex steroid hormones. The fact that triploid females showed no sign of developing secondary sexual characteristics (viz. shiny body colour and excessive

swelling of urogenital papilla) means that it may not only be the mechanical difficulties in random separation of chromosomes during the first meiotic division of eggs due to triploidy, but also the reduced serum vitellogenin and sex steroid hormone profiles. Similar explanation has already been reported for female triploids in other fishes (Lincoln and Scott, 1984; Nakamura et al., 1987; Lincoln and Bye, 1987; Benfey et al., 1987, 1989a).

The endocrine profiles (serum testosterone and 11-ketotestosterone levels) for males in *O. niloticus* did not show any dissimilarity between triploid and diploid groups at 45 weeks of age. This is consistent with the hypothesis that the triploid testis has enough steroidogenic cells functioning to produce abundant levels of sex steroids in fish (Scott, 1987). In spite of the functional sterility of triploid males,they exhibited all the physiological and morphological changes related to sexual maturation as diploid males (viz. bright and shiny body colour, bluish fin with redish margins and prominent urogenital papilla), which could be correlated with the production of sex steroid hormones by triploid males during their reproductive cycle. Similar endocrinological and morphological evidences on triploid males of other fishes have been presented by several workers (Lincoln, 1981a; Lincoln and Scott, 1984; Lincoln and Bye, 1987; Benfey et al, 1987; Benfey et al., 1989a).

In conclusion, the present data can be summarised with the hypothesis that triploid females are shown to be functionally and endocrinologically sterile because their germ cells can not continue the process of early meiotic division they produce less steroidogenic tissue and less hormone, therefore, they exhibit none of the endocrine changes relative to normal sexual maturation and ultimately no success in functional oocyte development. In contrast, triploid males are functionally sterile and although a few are able to produce spermatids leading to

aneuploid spermatozoa, they show normal endocrine profiles and develop testes very similar to those of diploids. Therefore it is apparent that the effect of triploidy on gonadal development and sexual maturation in Nile tilapia, *O. niloticus*, is greater in females than in males as in Atlantic salmon (Benfey et al., 1984b).

Despite the poorer growth performance, the complete suppression of ovarian development and sexual maturation of 3n females and functional and reproductive sterility of 3n male *O. niloticus* these fish may be of benefit to aquaculturists. Sterility is a potential factor for fish farming, where production cycles extends into maturity, stocking of these fish into production system would eliminate overpopulation and uncontrolled reproduction. In some case a better dressing percentage for the same weight of fish has been obtained (Chrisman et al., 1983; Lincoln and Scott, 1984; Krasznai and Marian, 1986). The impact of such reproductive sterility in mixed sex culture of *Oreochromis spp.* will be useful for improving production by preventing precocious sexual maturation and fertility of the growers particularly in ponds. The use of sterile triploid tilapia should also be the technique of choice if there is a risk of gene introgression of farmed stocks into native wild stocks. Moreover, though it is out of the scope of present study, it is commonly suggested that triploid induction could be coupled with sex inversion including tilapia such that all female, triploid fish could be produced for better monosex farming (Lincoln and Scott, 1983; Bye and Lincoln, 1986; Johnstone, 1989; Varadaraj and Pandian, 1990). The widespread use of all these techniques is limited at present until methods and commercial facilities to produce large number of triploid tilapia stocks at a reasonable cost can be established.

CHAPTER 4

GYNOGENESIS IN NILE TILAPIA, *OREOCHROMIS NILOTICUS* L.

GYNOGENESIS IN NILE TILAPIA, *OREOCHROMIS NILOTICUS* L.

4.1 INDUCTION OF MITOTIC GYNOGENETICS AND PRODUCTION OF THEIR CLONES IN *O. NILOTICUS* L.

4.1.1 Introduction

Gynogenesis is a form of artificial parthenogenesis where egg embryos develop as a result of activation by genetically inert spermatozoa. This means that embryonic growth is controlled by maternally inherited genes without any contribution from the male genome. In the process of induced gynogenesis, ova are fertilised by irradiated sperm which contain denatured DNA but are still motile and retain their ability to initiate embryonic development (Stanley and Sneed, 1974; Ijiri and Egami, 1980; Allen, 1987). The majority of embryos produced in this way are inviable haploids and die before hatching.

The number of viable gynogenetic progeny can be enhanced by artificial diploidization of the haploid maternal set of chromosome using a variety of physical shocks (temperature or hydrostatic pressure) and chemical treatments. These shocks may result in the retention of the second polar body of second meiotic division or the suppression of the first cleavage of mitotic division of eggs (for review see Purdom, 1983; Thorgaard, 1983; 1986; Chourrout, 1987; Nagy, 1987; Ihssen et al., 1990 and section 1.3 of this thesis). A schematic diagram of inducing gynogenesis in fish is given in Fig. 4.1.

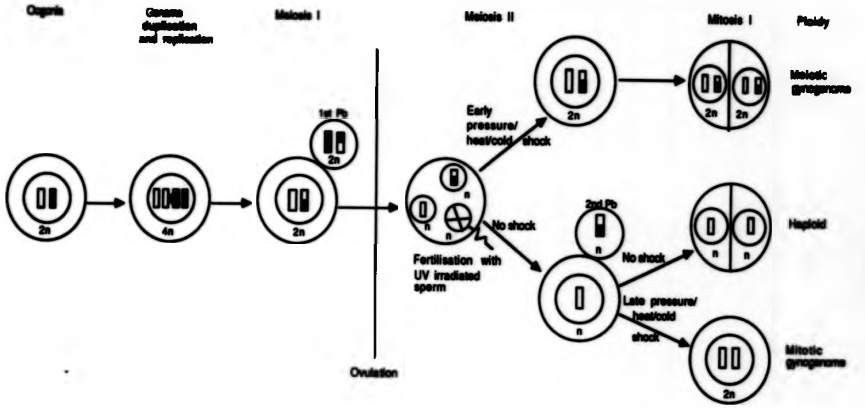


Figure 4.1. A schematic digram of inducing gynogenesis in fish using pressure, heat and cold shocks

The artificial induction of gynogenesis is a very promising technique for the aquatic sciences (Nagy, 1987). Gynogenesis by the suppression of meiotic cell division has been induced since 1960 in numerous fish species such as sturgeon, *Acipenser ruthenus* (Romashov et al., 1963), European loach, *Misgurnus fossilis* (Romashov and Believa, 1964, 1965), cyprinid loach (Suzuki et al., 1985b); common carp, *Cyprinus carpio* (Golovinskaya, 1968; Cherfas, 1975; Nagy et al., 1978; Nagy and Csanyi, 1982; Hollebecq et al., 1986; Linhart, et al., 1986; Taniguchi et al., 1986; Komen et al., 1988; Summantadinata et al, 1990), grass carp, *Ctenopharyngodon idella* (Stanley and Sneed, 1974; Stanley, 1976b); European catfish, *Silurus glanis* (Krasznai and Marian, 1987); plaice, *Pleuronectes platessa*, and flounder, *Platichthys flesus* (Purdom, 1969; Purdom and Lincoln, 1974; Purdom et al., 1976; Thompson et al, 1981); brown trout, *Salmo trutta* (Purdom, 1969); rainbow trout, *O. mykiss* (Chourrout, 1980; Chourrout and Quillet, 1982; Thorgaard et al. 1983; Chourrout, 1984; Lou and Purdom, 1984; Guyomard, 1984; Thompson and Scott, 1984; Kaastrup and Horlyck, 1987); brook trout, *Salvelinus fontinalis* (Arai et al., 1989); coho salmon, *Onchorhynchus kisutch* (Refstie, et al., 1982); chum salmon, *O. keta* (Onozatoo, 1982b); zebra fish, *Brachydanio rerio* (Streisinger et al., 1981); Pacific abalone, *Haliotis discus hannai* (Fujino et al., 1990); red sea bream, *Pagrus major* (Sugama et al., 1990a); tench, *Tinca tinca* (Linhart et al., 1991); Indian major carps, *Catla catla* (John et al., 1984); *Labeo rohita* (John et al., 1984; Dr. M.S. Shah, pers. communication); *Cirrhinus mrigala* (John et al., 1988; Dr. Shah, pers. communication) and tilapia, *Oreochromis* spp. (Chourrout and Itskovich, 1983; Penman et al., 1987b; Don and Avtalion, 1988b; Varadaraj, 1990).

Induced gynogenesis was considered a useful technique and has been widely applied in various genetic studies for estimating gene-centromere recombination, sex determination, rate of inbreeding, gene mapping distance and observing chromosomal rearrangement during evolution (Thorgaard and Allen, 1987; Nagy, 1987). In addition to these aspects, gynogenesis has made it possible to produce monosex populations in species in which the female parent was the homogametic sex (Golovinskaya, 1969; Nagy et al., 1978; Stanley, 1976; Refstie et al., 1982). At present there are few direct applications of gynogenesis in aquaculture because the fish are inbred and have a reduced variability compared to normal diploids. But the technique is of great potential use for producing unique gene combinations.

It has been commonly suggested that gynogenesis induction should be coupled with sex inversion such that functional XX all males could be produced (Nagy et al., 1981; Nagy 1987; Donaldson and Hunter, 1982; Jensen et al., 1983; Thorgaard and Allen, 1987). Such sex-reversed gynogenetic males are thought to be useful in crossbreeding experiments to produce all outbred monosex female population where the growth rate of females are superior to males particularly in salmonids and cyprinids .

Presently sex-reversed gynogenetic males are being widely used in China for line breeding and genetic improvement of common carp and some other commercial strains (Prof. Wu Chingziag, personal communication). There is evidence in *Oreochromis niloticus* that a spontaneously sex-reversed XY female and hormonally treated males can produce gynogenetically all male (YY) progeny (Scott et al. 1989). The main rationale for gynogenesis induction in fish, besides all the possibilities mentioned above, has been its

potential for generating rapidly inbred lines (Streisinger et al., 1981; Nagy and Csanyi, 1984; Nagy, 1987).

The utility of meiotic gynogenesis for the production of inbred lines depends on the frequency of recombination between any given gene and centromere during meiosis. Initially, it was expected that these gene-centromere crossing over frequencies would be very low in fish and a very high degree of homozygosity could be retained even in the first generation of meiotic gynogenetics (Nagy, 1987). Therefore, it was a common belief initially that meiotic gynogenetic offsprings might be useful for producing inbred lines (Golovinskaya, 1968; Purdom, 1969; cited by Ihssen et al., 1990). Golovinskaya and Romashov (1966) on the basis of their experimental results on common carp gynogenesis concluded that heterozygosity in gynogenetic diploids remains high because of the amount of crossing over in the region between genes and the centromere. The application of electrophoretic markers in several studies revealed that the rate of crossing over in the first meiotic metaphase generates high levels of heterozygosity in chromosome regions distant from the centromere (Purdom et al., 1976; Cherfas, 1977; Cherfas and Truweler, 1978; Nagy et al., 1978; Thompson et al., 1981; Streisinger et al., 1981; Nagy and Csanyi, 1982; Thorgaard et al., 1983; Thompson, 1983; Gervai and Csanyi, 1984; Thompson and Scott, 1984; Guyomard, 1984, 1986; Allendorf et al., 1986).

On the basis of the results of electrophoretic loci analyzed to date (see section 1.3), most of the above workers suggested that gynogenesis involving retention of the second polar body is not an efficient way of producing homozygous inbred lines as was previously expected. Although as an alternative step, repeated heterozygous gynogenetic reproduction

may lead to genetically identical strains these will not be homozygous at every locus (Nagy and Csanyi, 1984; Ihssen et al., 1990). Han et al. (1991) suggested that homozygous inbred lines will never be produced by using meiotic gynogenetic diploids, even when reproduction is repeated for several generations. Purdom and Lincoln (1973) pointed out that to produce inbred lines by conventional methods of sib-mating requires the maintenance of several lines with close inbreeding for up to 20 generations. But gynogenesis especially by inhibiting first mitotic division could dramatically shorten the time required to produce completely homozygous progeny in the first generation and an "inbred line" or "clone" in the second.

The gynogenetic diploid produced by the suppression of first cleavage of eggs (designated as mitotic gynogenetics) is considered to be more useful than the meiotic type for the fixation and establishment of a new race of fish in aquaculture (Taniguchi et al., 1988). The induction of homozygous diploid gynogenetics in zebra fish, *Brachydanio rerio*, in an attempt to produce their clones was first successfully demonstrated by Streisinger et al., 1981). Subsequently the technique has been applied to other fish species (Chourrout, 1984; Onozato, 1984; Purdom et al., 1985; Naruse et al., 1985; Ijiri, 1987; Krasznai and Marian, 1987; Nagy, 1987; Taniguchi et al., 1988; Komen et al., 1991a; Han et al., 1991; Quillet et al., 1991; Gouidie et al., 1991). Despite the fact that mitotic gynogenesis has been induced in many fishes, until recently clones have only been produced in *Brachydnio rerio* (Streisinger et al., 1981); *Oryzias latipes* (Naruse et al., 1985; Ijiri, 1987); *Cyprinus carpio* (Komen et al., 1991) and *Plecoglossus altivelis* (Han et al., 1991). Clones are potentially very valuable products for selective breeding programmes and improvement of fish stock

(Han et al., 1991) but the likelihood of practical application of such lines in fish culture and management remains uncertain due to lack of follow up trials (Thorgaard and Allen, 1987).

As discussed elsewhere (Chapter 3) tilapias are already subject to many different genetic manipulations to improve their utility in aquaculture. In addition to these developments in tilapia genetic research, the production of clonal lines by cross-breeding of two completely homozygous individuals and gynogenetic technique in this species is expected to contribute greatly to the development of a new breeding strategy and genetic improvement of the stock. The only published work on induction of mitotic gynogenetics in *O. niloticus*, by heat shock was that of Mair et al. (1987) but no further attempts at producing clones in this fish have so far been reported.

As a first step in the development of clones of *O. niloticus* the present study aimed to identify the optimum pressure and heat shock treatments to induce viable mitotic gynogenetics by the suppression of first cleavage in eggs fertilised with UV irradiated milt. Offspring from these treatments were analyzed using karyological and electrophoretic techniques to confirm their genetic status and to compare the differences between the techniques. In the subsequent trials, heterozygous and homozygous clones of the fish have been successfully produced using reproductively viable mitotic gynogenetic broods. The results obtained from these experiments are presented and discussed.

4.1.2 Materials and methods

4.1.2.1 Origin of fish stock:

The *O. niloticus* 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). All broodstock were grown up to maturity stage under proper maintenance (section 2.1.2) and feeding (section 2.1.3).

4.1.2.2 Collection and UV treatment of sperm:

Fresh sperm was collected (section 2.5) from sexually matured males for every experiment. Sperm samples requiring UV treatment were checked for motility and irradiated (section 2.5) with an ultraviolet lamp set using a radiometer (Ultra-Violet Products Inc.).

4.1.2.3 Collection and fertilisation of ovulated eggs:

Eggs were collected by gently stripping ovulated female(s) (section 2.2). Once stripped, each batch of eggs was fertilised *in vitro* either with normal or UV treated sperm as explained below.

4.1.2.4 Experimental designs for induction of gynogenetics and production of their clones:

Meiotic gynogenomes were produced by exposing eggs fertilised with UV irradiated sperm to the optimal second polar body retaining pressure (8000 p.s.i. for 2 mins. at 9 mins. a.f.) and heat shock (41 °C for 3.5 mins. at 5 mins. a.f.) treatments as identified earlier (see

section 3.3.1). To interfere with the first mitosis the following parameters of pressure and heat shocks were used: Pressure shocks of 8000 - 10,000 p.s.i. by 500 p.s.i. steps; durations of 1-4 mins. by 1 min. increments applied 25 - 65 mins. a.f. by 2.5 mins. increments; Heat shocks of 40 - 42 °C by 1 °C steps; durations of 2.5 - 5.5 mins. by 1 min. increments applied 20 - 55 mins. a.f. by 2.5 mins. increments. All the treatment batches of eggs were fertilised by mixing 0.4 - 0.5 ml diluted (with modified Cortland's solution) UV irradiated sperm, un-irradiated sperm from the same pool was used to fertilise a portion of eggs as a control. After fertilization, when not being submitted for shock treatments, eggs were at all times incubated at 28±1°C (section 2.4). All treatment batches of eggs except the UV control were exposed to elevated hydrostatic pressure and heat shocks as described in the section 2.3.

A total of 20 females heterozygous at either or both *ADA** and *EST-2** loci were used in the optimisation trials of both pressure and heat shock treatments, some females were used more than once but in different experimental series.

The aim of this study was to identify treatment optima for the suppression of first cleavage during mitotic division of eggs by altering the relevant parameters of pressure and heat shocks. Thus attention was first focused on the effective timing of application of pressure or heat treatments (on set of shock). Logistically for long time series for time after fertilisation in pressure and heat shocks, it was easier to use two sets of eggs. So that each treatment could be done on alternative sets. This meant that subsequent treatments every 2.5 minutes occurred every 5 minutes on a given set from 25 - 65 mins. a.f. for pressure

and 20 - 55 mins. a.f. for heat. When a.f. was established then shock duration and intensity could be optimised.

All survival rates were estimated on the basis of incidence of normal fry at hatching and yolk sac resorption. Fry were also analyzed karyologically particularly at hatching and the results presented as percentages of normal diploid compared to internal diploid control which was set at 100%. Logistically it was impossible to do all treatments on every batch but between 3 - 5 different females (as replicates) were used to analyze all parameters tested. Results are presented as means of all replications with their standard errors.

Two sexually matured reproductively viable mitotic gynogenetic females, both homozygous at the *ADA** locus for 113/113 (slower) allele and two mitotic gynogenetic males (recessive mutation in a sex determining gene), both homozygous *ADA** locus for 135/135 (faster) allele were used as parents for the production of outbred (OCL) and inbred (ICL) clonal lines . Eggs stripped from each of the female fish were divided into five equal batches (ca 200 - 250 eggs per batch). The first, second and third batches of eggs were fertilised with UV irradiated (section 2.5) sperm (pooled milt of two males). The first batch of fertilised eggs was left as UV control but the second and third batches were exposed respectively either to early pressure (a shock approximately 8000 p.s.i. for 2 min at 9 mins. a.f.) or early heat (41±0.2 ^{o}C for 3.5 mins. at 5 mins. a.f.) shocks. The fourth and fifth batches of eggs were fertilised separately with intact (un-irradiated) sperm of two mitotic gynogenetic males to produce two clonal families involving two different females. In each experiment, survival rates of ICL line at four embryonic stages (section 2.4) are estimated as means of two

replicated treatments. All survival rates of OCL line are the means of two normal crosses involving two different mitotic males.

4.1.2.5 Egg incubation and checking of survival rates:

All treated and untreated batches of eggs were identically incubated (section 2.4) and the survival rate of embryos in each batch was checked and calculated at four developmental stages such as morula (MOR); pigmentation (PIG); hatching (HAT) and yolk sac resorption (YSR) as (number of embryos survived at a given development stage/original number of eggs) x 100. The number of normal and deformed fry at both HAT and YSR stages were recorded carefully.

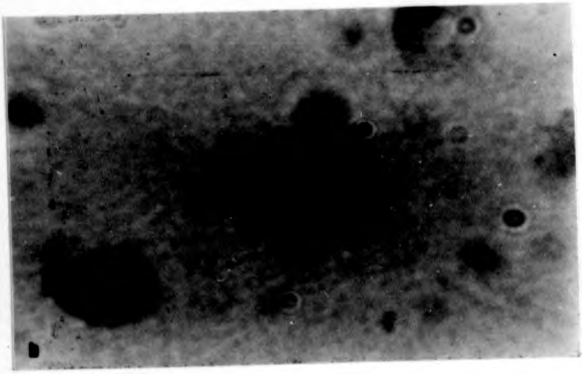
4.1.2.6 Determination of ploidy:

The ploidy status of newly hatched or 1 day old larvae of meiotic, mitotic and control progeny was determined by karyotypic analysis of several (>3) karyotypes per individual and 5 - 10 individuals (sub-sample of both normal and abnormal fry) per treatment using a modified technique as described earlier (section 2.7.1). Haploid and diploid metaphases were composed of one (N=22 including one large marker chromosome) and two (N=44 including two marker chromosomes) set of chromosomes respectively (Fig. 4.1.2.6). Haploid/diploid mosaics were identified by counting the abnormal chromosome numbers (between N=22 and 2N=44) associated with either one or two large marker/sub-metacentric chromosomes.

Figure 4.1.2.6

(a) Metaphase chromosome spread of haploid (n = 22) individual in *O. niloticus*. One large marker chromosome is indicated by arrow.

(b) Metaphase chromosome spread of diploid (2n = 44) individual in *O. niloticus*. Two marker chromosomes are indicated by arrows.



4.1.2.7 Electrophoretic analysis:

The starch gel electrophoresis (section 2.8) of biopsied tissues was used to determine the genotype of all the broodstock and progeny in this series of experiments on gynogenesis.

a) Selection of broodstock: Blood and fin tissues were collected from a part of sexually mature *O. niloticus* in total 70 females and 35 males were sampled. The biopsies were analyzed for 6 polymorphic loci (McAndrew and Majumdar, 1983; Shaklee et al., 1990; Panom Sodsook pers. communication) as follows:

- *ADA** (Adenosine deaminase, ADA. EC No. 3.5.4.4) with three alleles (135, 121 and 113);
- *EST-2** (Esterase, EST. EC No. 3.1.1.1) with two alleles (105 and 100);
- *AAT-2** (Aspartate aminotransferase, AAT. EC No. 2.6.1.1) with two alleles (100 and 80);
- *MEP-2** (Malic enzyme-NADP, MEP. EC NO. 1.1.1.40) with two alleles (110 and 100);
- *AH** (Aconitate hydratase, AH. EC No. 4.2.1.3) with two alleles (100 and 85);
- *FH-1** (Fumarate hydratase, FH., EC No. 4.2.1.2) with two alleles (107 and 100).

b) Fin biopsies from 2 - 3 month old meiotic, mitotic and control sibs belonging to 4 different progeny groups/families produced by pressure and heat shocks were collected and tested electrophoretically to determine their genotype frequency at the *ADA** locus. The electropherogram were designed to show the ADA banding pattern of mitotic, meiotic and control progeny group together with their parental genotype. fin samples of 5 progeny individuals of a representative family and each of the parents were collected and stained for starch gel accordingly.

c) Several tissues such as blood, muscle, liver and kidney were collected at 33 weeks of age from 70 meiotic gynogenome progeny derived from a heterozygous (at six loci to be tested for progeny) female parent in an attempt to measure the frequency of heterozygotes or gene-centromere recombination. These were compared to samples collected from 20 mitotic and 24 control sibs. The tissues analyzed and the enzymes stained were: muscle for *ADA**, *AAT-2**, *MEP-2**, *FH-1**; liver for *AAT-2**, *AH**; kidney for *AAT-2**, *AH**; blood for *EST-2**.

d) A total of 12 mitotic females and 6 males were selected as broodstock for the production of clonal lines. Biopsied fin samples of these broodstock were collected and tested at *ADA** and *EST-2** loci to ascertain their genetic and allelic status.

e) *ADA** has been found the most useful marker for identifying gynogenetic fish. In the present study muscle samples from a total of 80 outbred (OCL) and 7 inbred (ICL) progeny (30 days old) from two different families were collected and tested at the *ADA** locus. The mobility of the alleles were checked against parental and full sib samples.

4.1.3 Results

4.1.3.1 Suppression of first cleavage - a comparison of relative performance of pressure and heat shocks to induce mitotic gynogenetics:

Table 4.1.3.1A shows genotypes of all the female and male broodstock electrophoretically screened at two loci *ADA** and *EST**. A total of the 57.1% of the females and 71.4% of the males were heterozygotes for *ADA**. At the *EST-2** locus 21.43% of the females and 34.29% of the males were heterozygotes. Among all the heterozygous parents eight females and nine males were heterozygotes at both the loci. Among heterozygous parental stock particularly the female parents were selectively used to collect eggs for the proposed gynogenetic experiments in this study.

The mean percent survival to hatch (Fig. 4.1.3.1A) and yolk sac resorption (Fig. 4.1.3.1B) of normal fry (putative mitotic gynogenetics) induced by the most effective late pressure shock intensity (9000 p.s.i) and duration (2 mins.) applied at various timings (25 - 65 min a.f.), are presented. The results of various durations (1 - 4 mins.) of the optimum pressure shock and timing are also presented sequentially from front to back on the "Z" axes in the same figures. The optimum early pressure shock (8000 p.s.i.) and duration (2 mins.), which was found maximally effective at inhibiting the extrusion of second polar body (Chapter 3), was ineffective when applied on batches of egg from 25 to 65 mins. a.f. in suppressing first cleavage. Higher pressures (9500 - 10,000 p.s.i.) were also ineffective and have not been included in Fig. 4.1.3.1A and Fig. 4.1.3.1B. An intermediate pressure level (9000 p.s.i.) was found to be the most effective at inducing viable mitotic gynogenetic progeny

Table 4.1.3.1A. Observed *O. niloticus* broodstock genotypes at *ADA** and *EST-2** loci. Values calculated in percentage are shown within parentheses. F = Female; M = Male.

Enzyme loci	Sex	Fish no.	Broodstock genotypes (%)				
*ADA**			135/135	135/113	135/121	121/121	113/113
	F	70	16(22.9)	33(47.1)	7(10.0)	-	14(20.0)
	M	35	5(14.3)	22(62.8)	3(8.6)	2(5.7)	3(8.6)
*EST-2**			105/105		105/100		100/100
	F	70	5(7.1)		15(21.4)		50(71.4)
	M	35	2(5.7)		12(34.3)		21(60.0)

when given in the period of 40 - 50 mins. a.f. at 28±1°C. The optimum duration of pressure shock (9000 p.s.i.) at maximally effective timing (47.5 mins. a.f.) was 2 mins. Any shorter or longer durations were not exactly effective. Earlier on set of shock (25 - 32.5 mins. a.f.) and later (55 - 65 mins. a.f.) did not induce any mitotic gynogenetics.

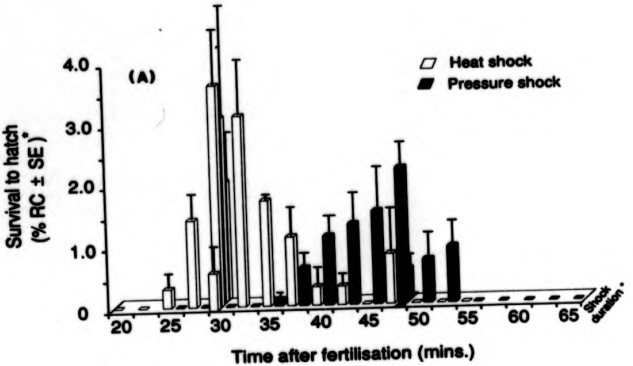
All UV control batches showed a sharp reduction in embryonic survival from the pigmentation stage (45 - 50 h a.f.). Typically fry had twisted small bodies with a watery sac (haploid syndrome) and all died between the period of hatch and yolk sac resorption. In contrast, the controls (eggs fertilised with normal sperm) at all times showed high survival rates (79.0%±3.5, N=10 at hatching and 70.5%±6.5, N=10 at yolk sac resorption) with a low number of deformed fry.

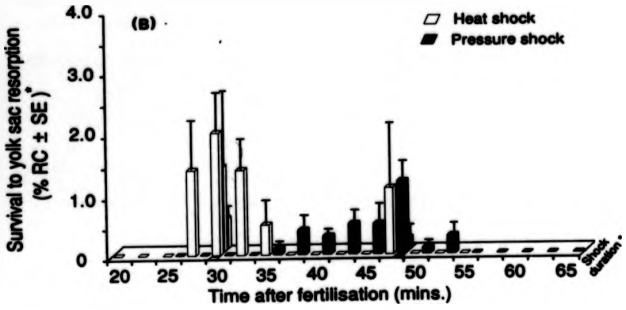
The results of the various timings of shock on set (20 - 55 mins. a.f.) of the most effective heat shock at varying durations (Z axis 2.5 - 5.5 mins.) on the subsequent survival and incidence of normal fry (putative mitotic gynogenetics) at hatching and yolk sac resorption are presented graphically in Fig.4.1.3.1A and Fig. 4.1.3.1B.

As with meiotic gynogenesis the optimal temperature shock of 41±0.2°C was found highly effective at inducing suppression of first cleavage in eggs between 27.5 - 30 mins. a.f. Temperature shocks above (42 °C) and below (40 °C) this level resulted in no viable mitotic gynogenetic survival and have not been included in Fig. 4.1.3.1A and Fig. 4.1.3.1B. At 41±0.2°C the most effective shock duration was 3.5 mins. when administered at 30 mins. a.f. Earlier timings (20 - 25 min a.f) at this level of intensity and duration did not produce any mitotic gynogenetics. Later timings (37.5 - 55 min a.f.) showed similar results except

Figure 4.1.3.1 (A - B)

The application of heat (41 °C x 3.5 mins.) and pressure (9000 p.s.i. x 2 mins.) shocks at different times after fertilisation to *O. niloticus* eggs on the subsequent survival and incidence of normal fry (putative mitotic gynogenetics) at (A) hatching and (B) yolk sac resorption. *RC relative to control survival rates. The results of heat and pressure shock durations (see Materials and Methods) at 30 and 47.5 mins. a.f. respectively are presented sequentially from front to back on the "Z" axes.


(A)
Heat shock
Pressure shock
Survival to hatch
(% RC ± SE)
4.0
3.0
2.0
1.0
0
20
25
30
35
40
45
50
55
60
65
Shock duration
Time after fertilisation (mins.)


(B)
Heat shock
Pressure shock
Survival to yolk sac resorption
(% RC ± SE)
4.0
3.0
2.0
1.0
0
20
25
30
35
40
45
50
55
60
65
Shock duration
Time after fertilisation (mins.)

the 47.5 min a.f., where a second peak of mitotic gynogenetics induction and survival was observed. In this study, all control batches of egg had good survival rates of normal fry at hatching (64.6%±7.0, N=10) and at yolk sac resorption (49.4%±6.4, N=10). No survival of normal fry was recorded in all the UV control batches of eggs.

Karyological analysis of all mitotic gynogenetics produced, using the optimum shock intensities and durations for pressure and heat, showed that the window for the successful induction of mitotic gynogenetics was narrower than the survival data would suggest. A high frequency of haploid/diploid mosaics was observed either side of the 40 - 50 mins. a.f. for the pressure shock and either side of 27.5 - 30 mins. a.f. for heat shock.

The electrophoretic genotypes at the *ADA** locus in four different progeny groups/families (full-sib controls, meiotic and presumed mitotic gynogenetics) derived from four different heterozygous females are shown in Table 4.1.3.1B. A gel showing the *ADA** banding pattern made up from the three progeny groups in a single family is shown in Fig. 4.1.3.1C. The result show that all 72 samples of meiotic gynogenetic progeny had the same genotype as the heterozygous female parent. In contrast all 44 samples belonging to 4 families of mitotic gynogenetic progeny were completely homozygous for one or other of the maternal alleles. The genotypic distribution in 76 control sib samples were not significantly different ($P>0.05$) from Mendelian ratios expected from the genotypes of the parents. No paternal inheritance was observed in any of the UV sperm treated batches within these 4 progeny families.

Table 4.1.3.1B. Genotypes observed at the Ada- locus in the progeny from four different families, each family being split into three groups: mitotic gynogenetics, meiotic gynogenetics and normal controls. MITP = pressure shock mitotic gynogenetics; MITH = heat shock mitotic gynogenetics; MEIP = pressure shock meiotic gynogenetics; MEIH = heat shock meiotic gynogenetics; CON = control. Numbers in parentheses are expected numbers of genotypes.

Parents		Progeny		Progeny genotype						Chi-square
Female	Male	Type	Number	135/135	135/121	135/113	121/121	121/113	113/113	
135/121	135/113	MITP	5	3	0	0	2	0	0	–
		MEIP	12	0	12	0	0	0	0	–
		CON	12	3(3)	4(3)	3(3)	0	2(3)	0	0.88 NS
135/113	135/135	MITP	5	1	0	0	0	0	4	–
		MEIP	10	0	0	10	0	0	0	–
		CON	12	5(6)	0	7(6)	0	0	0	0.56 NS
135/113	121/113	MITH	4	2	0	0	0	0	2	–
		MEIH	20	0	0	20	0	0	0	–
		CON	20	0	4(5)	6(5)	0	5(5)	5(5)	0.94 NS
135/113	135/121	MITH	30	16	0	0	0	0	14	–
		MEIH	30	0	0	30	0	0	0	–
		CON	32	6(8)	8(8)	9(8)	0	9(8)	0	0.86 NS

NS = Not significant

Figure 4.1.3.1 (C)

A gel showing the *ADA** banding pattern of control, meiotic and mitotic gynogenetic progeny groups. The genotypes at the *ADA** locus are shown below.

MITOTIC
MEIOTIC
CONTROL
135/135
113/113
135/135
113/113
135/113
121/113
135/113
135/121
135/113
135/113
135/121

4.1.3.2 Gene-centromere recombination rate at various enzyme loci in meiotic and mitotic gynogenetic progeny:

The distribution of genotypes in meiotic and mitotic gynogenetic progeny derived from a female heterozygous at *ADA**, *EST-2**, *AAT-2**, *MEP-2**, *AH** and *FH-1** loci are presented in Table 4.1.3.2. It is clear from all mitotic progeny that the mother's alleles are only expressed in the homozygous condition at all loci. However in the case of the meiotic progeny no heterozygotes were observed at the *EST-2** locus but all progeny were heterozygotes at the *ADA** locus. The frequencies of such heterozygotes at other loci were, *AAT-2** (52.9%, N = 70), *AH**(45.7%, N = 70), *MEP-2** (25.7%, N = 70) and *FH-1**(20%, N = 15). Therefore, the average proportion of heterozygotes (y) in meiotic progeny at six enzyme loci was y = 0.41. No significant deviation was observed from the expected Mendelian ratios in all control sib samples (N = 24) at polymorphic loci analyzed in this study,although the results are not included in the Table.

Table 4.1.3.2. Distribution of genotypes and proportion of heterozygotes (y) at six loci in meiotic and mitotic gynogenetic progeny derived from heterozygous (at all loci) female parent. Meio.= Meiotic gynogenome progeny; Mito.= Mitotic gynogenome progeny.

Enzyme loci	Female parent genotype	Progeny		Progeny genotypes[1]			Proportion heterozygotes (y)
		Type	No.	F/F	F/S	S/S	
*ADA**	135/113	Meio.	60	0	60	0	1.00
		Mito.	20	10	0	10	0.00
*EST-2**	105/100	Meio.	30	8	0	22	0.00
		Mito.	20	12	0	8	0.00
*AAT-2**	100/80	Meio.	70	21	37	17	0.53
		Mito.	7	4	0	3	0.00
*MEP-2**	110/100	Meio.	70	25	18	27	0.26
		Mito.	7	3	0	4	0.00
*AH**	110/85	Meio.	70	13	32	25	0.46
		Mito.	7	3	0	4	0.00
*FH-1**	107/100	Meio.	15	0	3	12	0.20
		Mito.	7	4	0	3	0.00

[1]F/F = homozygous for faster allele of maternal genotype;
F/S = heterozygous for both faster and slower alleles of maternal genotype;
S/S = homozygous for slower allele of maternal genotype.

4.1.3.3 Production of heterozygous and homozygous clones of *O.niloticus*:

Table 4.1.3.3A shows the observed electrophoretic genotypes of 12 selected mitotic females (MF) and 6 mitotic males (MM) derived from a female heterozygous at *ADA** and *EST-2** loci. All mitotic progeny were homozygous for one or another of the two maternal alleles. No inheritance of paternal genotype was observed in any of the broodstock examined.

The two most fecund and reproductively proven mitotic females were used as broodstock for the production of outbred and inbred clones. The data of different clonal lines at four embryonic development stages (section 2.4) are plotted in Fig. 4.1.3.3A and Fig. 4.1.3.3B. In Expt. 1, eggs of mitotic female MF-1 were fertilised with normal (untreated) and UV irradiated sperm of two mitotic males MM-1 and MM-3 and the latter was exposed to early shock treatments (pressure/heat). The mean fertilisation rate of outbred clone (OCL) group scored at MOR stage was 87.1%. For which the mean survival rate (relative to OCL) of inbred clone (ICL) and haploid (HAP) groups was 85.7% and 84.0% respectively. All embryos in HAP group died before HAT stage. A sharp reduction of mean embryonic survival occurred in the ICL group from PIG until YSR. In contrast the OCL group showed a gradual reduction in mean survival rate at all these embryonic stages. A similar trend of embryonic survival at four development stages was also observed for the three treatment groups in Expt.2 involving eggs of mitotic female MF-2 fertilised with sperm of the same two mitotic males. The survival rate of normal fry of OCL at first feeding (YSR stage) in Expt.1 and Expt.2. was recorded as 40.7%±7.2SE and 25.1%±2.3SE respectively. At first feeding, the survival rate (relative to OCL) of normal fry in the ICL group was 4.3%±0.02SE and 4.8%±1.12SE in Expt.1 and Expt.2 respectively. A high frequency of

Table 4.1.3.3A. Genotypic status at *ADA** and *EST-2** loci of some mitotic broodstock selected for checking their fertility and using for the production of clonal lines. MF = Mitotic Female; MM = Mitotic Male.

Parents			Mitotic broodstock		Mitotic broodstock genotypes*		
Female	Male	Loci	Sex	Number	F/F	F/S	S/S
135/113	135/121	*ADA**	MF	12	5	0	7
			MM	6	4	0	2
105/100	105/100	*EST-2**	MF	12	3	0	9
			MM	6	2	0	4

*F/F = homozygous for faster allele of maternal genotype;
F/S = heterozygous for both faster and slower alleles of maternal genotype;
S/S = homozygous for slower allele of maternal genotype.

Table 4.1.3.3B. Segregation of genotypes at *ADA** locus in the progeny from two different clonal families. Each family split into two groups: Outbred clone (OCL) and Inbred clone (ICL).

Clonal family	Parental genotypes		Clonal progeny		Clonal progeny genotypes		
	Female	Male	Type	Number	113/113	135/113	135/135
1	113/113	135/135	OCL	50	0	50	0
			ICL	4	4	0	0
2	113/113	135/135	OCL	30	0	30	0
			ICL	3	3	0	0

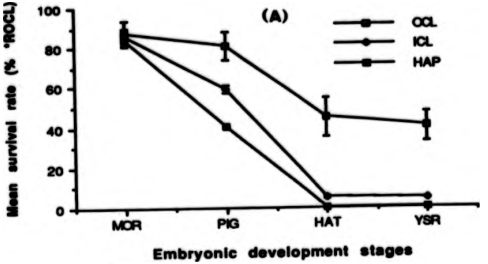
deformed fry were also recorded but they died before or just after YSR stage in the ICL group.

The outbred and inbred nature of two types of clonal lines produced in two families were checked and identified at the highly polymorpic *ADA*^{a} locus and the results of segregation of their genotypes are presented in Table 4.1.3.3B. Fig. 4.1.3.3C shows the *ADA*^{a} banding pattern in electropherogram representing pooled samples from two families of outbred (OCL) and inbred (ICL) clones including samples from their homozygous parents. The progeny samples of all 80 OCL (outbred clone) derived from 4 different conventional crosses between mitotic females and males were all heterozygotes at this particular locus and the pattern of genotypes were identical to the two homozygous alleles inherited from their female and male parents. In contrast genotypes of all the representative samples of gynogenetic ICL (inbred clone) were homozygous and identical to their maternal allele.

Figure 4.1.3.3 (A - B)

Showing results of producing outbred (OCL) and inbred (ICL) clonal lines in *O. niloticus* and viability of their survival at four embryonic development stages in Expt.1 (A) and Expt.2 (B). MOR = Morula; PIG = Pigmentation; HAT = Hatching and YSR = Yolk sac resorption; HAP = Haploid control.

***ROCL = Relative to outbred clone.**


(A)
OCL
ICL
HAP
Mean survival rate (% *ROCL)
100
80
60
40
20
0
MOR
PIG
HAT
YSR
Embryonic development stages

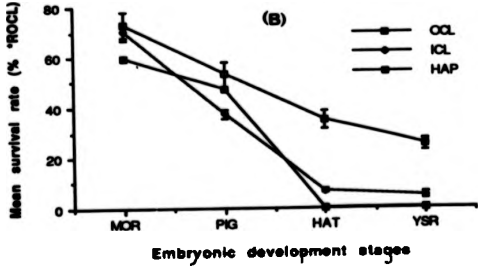
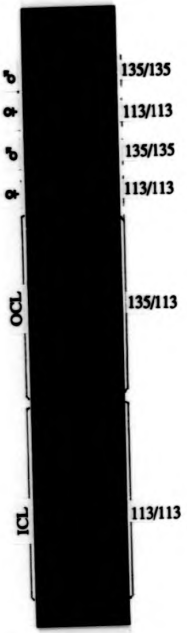

(B)
OCL
ICL
HAP
Mean survival rate (% *ROCL)
80
60
40
20
0
MOR
PIG
HAT
YSR
Embryonic development stages

Figure 4.1.3.3 (C)

Electropherogram showing *ADA** banding pattern of representative samples from two families of outbred (OCL) and inbred (ICL) clones including their mitotic gynogenetic parents. Genotypes at the *ADA** locus are shown below the gel.


♂
♀
♂
♀
OCL
ICL
135/135
113/113
135/135
113/113
135/113
113/113

4.1.4 Discussion

4.1.4.1 Mitotic gynogenetic induction by late pressure and heat shock treatments:

In this study, it was clearly revealed that the pressure level for the successful inhibition of mitotic events was higher (9000 p.s.i.) than that required for meiotic events (8000 p.s.i), but the effective duration was the same (2 mins.). The result is similar to that reported by Taniguchi et al. (1988) for ayu where a higher pressure was necessary to disrupt the mitotic spindle over that required for meiotic shocks. (1988). The only other work on suppression of first cleavage in *Oreochromis* species using hydrostatic pressure was an attempt to induce tetraploidy by Myers (1986). He used a combination of pressure and cold shocks (6500 - 7500 p.s.i at 7.5 °C) to produce tetraploids although it appears from and present study (section 3.3.1.3) that this temperature of cold shock with sufficient duration and or a higher pressure alone may have been sufficient. Don and Avtalion (1988b) demonstrated that a cold shock of 11 °C for 60 mins. duration alone could successfully induce tetraploidy in *O. aureus*.

The results of heat shock application for the suppression of first cleavage in *O. niloticus* was essentially similar to that of Mair et al. (1987). They used a single temperature (41 °C) and duration (3.5 mins.) and their optimal a.f. range was based mainly on survival to pigmentation rather than hatching or yolk sac resorption. This study would suggest that their range of 25 - 35 mins a.f. may be too wide, mosaicism at the extremes serving to narrow the effective treatment "window". The 27.5 - 30.5 min a.f. window observed in the present study involving *O. niloticus* gave only diploids and very few haploids at HAT and YSR.

In common carp, *Cyprinus carpio*, both Nagy (1987) and Komen et al. (1991a) also found a narrow effective "window" (28 - 30 mins. a.f.) for the production of mitotic gynogenomes by heat shock despite working at two different temperatures. The success of inducing viable homozygous gynogenesis by physical shock treatments mainly depends on synchronous development of eggs in conjunction with proper timing of the first mitosis (Streisinger et al., 1981; Chourrout, 1984; Naruse et al., 1985 cited by Komen et al. 1991a).

In fish species there is little information on the time scale of cytological development in eggs. In common carp,*cyprinus carpio*, Komen (1991a) has recently examined histologically the developing embryos during the first mitotic division at 24 °C between 20 to 40 mins. a.f. Komen saw cytological evidence of first cleavage from 25 - 35 mins. a.f. and timing to inhibit first mitosis at 25 mins. a.f. was earlier. Therefore, the most effective timing to induce suppression of first cleavage by heat shock in common carp was at 30 mins. a.f. Obviously different fish species have different rates of cytological development during embryogenesis. From Fig. 4.1.3.1A and Fig 4.1.3.1B, it was observed that the late effective window and the width of timings 40 - 50 min a.f. at 28 °C was about to be optimum for maximum interference of mitotic events using pressure shocks to induce viable putative mitotic gynogenomes in the present species. These timings were around 50 -55 % (42.5 - 49.5 mins. a.f.) of time to cleavage as estimated on the basis of investigation carried out to search for the timings of first cleavage in *O. niloticus* eggs in the present study (section 2.6). This cleavage timing is intermediate between the timings reported by Myers (1985) and Mair (1988) in the same fish at the same temperature. The late effective windows clearly suggests that the restoration of diploidy in these embryos is at prometaphase-metaphase stage of first mitosis.

The 10 mins. (40 - 50 mins. a.f.) window for pressure is probably a result of developmental asynchrony in any given batch of eggs. This would suggest that it's only eggs at the "sensitive stage" which are affected in the different a.f. shocks given. The development of eggs that should enter this stage during the shock must be halted otherwise they would receive a sub-optimal shock and so display mosaicism. It would appear that the optimal shock will stop normal egg development whereas the various combinations of sub-optimal shocks will not. Mosaicism can be explained as suppression of cell cleavage at the wrong stage of metaphase which leads to partial disruption of microtubles of the mitotic spindle.

Closer inspection of optimization of the present results shows that the timing of initiation of heat shock for suppression of first cleavage is earlier than pressure shock, because the higher temperature has the effect of advancing the rate of all biological processes. This will make the timing of shock application even more critical, as the width of effective window will be narrower, than for the corresponding pressure shocks. A similar hypothesis has been advanced to explain the effects of heat and pressure shocks to induce retention of the second polar body for triploid production in Nile tilapia (see Chapter 3). Some earlier studies have shown that pressure shocks often result in improved survival compared to temperature shocks. This was the case for the meiotic gynogenetics produced in this study where at the selected optimum the overall survival for pressure was 24.0%±7.5, N=10 and that for heat was 13.6%±4.5, N=10 at YSR. Pressure and heat were found equally successful in inhibiting mitotic processes. No significant differences in survival were observed at YSR for the mitotic gynogenetics of pressure (1.2%±0.3SE, N=5) and heat (2.0±0.6SE, N=5). However, it might be argued that pressure gives a wider window for successful induction of mitotic gynogenetics and therefore less risk of mosaicism.

It is the very low survivals of mitotic gynogenetics which is inhibiting the mass production of these individuals in the present fish. Recessive lethal or deleterious genes in homozygous fry might be the reason for such a reduction in gynogenetic survival (Onozato, 1984; Lou and Purdom, 1984b; Purdom et al., 1985; Mairs et al., 1987). Although Streisinger et al. (1981); Komen (1991a) and Quillet et al. (1991) attained comparatively higher survival frequencies of mitotic gynogenetic fry respectively in zebra fish (20%), common carp (max. 15.7%) and rainbow trout (16%). This phenomenon might therefore be species specific.

Electrophoretic comparison of the mitotic and meiotic gynogenetics with the controls showed no evidence of paternal inheritance and therefore cofirmed the success of the UV treatment of the milt. At the *ADA** locus, only homozygotes in all mitotic gynogenetic progeny induced by optimal late pressure and heat shocks confirmed that diploidisation was caused by the suppression of first cleavage. In contrast only heterozygotes appeared in all samples of meiotic gynogenetic progeny providing evidence of 100% recombination and therefore, restoration of diploidy occurred by the retention of the second polar body. The only other report was by Mair (1988), who compared control with heat shocked meiotic and mitotic progeny at *ADA** locus and observed a similar pattern of genotypic segregation in his samples of the same species.

4.1.4.2 Proportion of heterozygotes and degrees of inbreeding in meiotic and mitotic gynogenetic progeny:

Gene-centromere recombination frequency has been estimated in this study at six enzyme loci such as *AAT-2**, *EST-2**, *MEP-2**, *AH** and *FH-1** including *ADA** in a different experiment involving both meiotic and mitotic progeny derived from a single female *O.*

niloticus heterozygous at all the loci. All mitotic gynogenome progeny showed no recombination between the respective gene and centromere at six loci. In contrast recombination frequency in meiotic progeny was 0 - 100%. Therefore, the varying proportion of heterozygotes (y) were observed at *EST-2** = 0, *FH-1** = 0.20, *MEP-2** = 0.26, *AH** = 0.46, *AAT-2** = 0.53 and *ADA** = 1.0 (Table 4.1.3.2) suggesting that this is probably a reflection of the relative position of the various loci to the centromere on their respective chromosomes. As the mean proportion of heterozygotes (mean y = 0.41) is known it is easy to calculate the degrees of inbreeding using the equation F = (1 - mean y)(Allendorf and Leary (1984). Therefore, F = 0.59 in meiotic gynogenetic progeny of the present fish. This value for *O. niloticus* is comparable with the degree of inbreeding (F) already estimated in meiotic gynogenetics of other fish such as in carp (F = 0.65; Nagy et al., 1982); plaice (F = 0.58, Thompson, 1983); rainbow trout (F= 0.32; Guyomard, 1984; F = 0.44, Allendorf, et al., 1986) and red sea bream (F = 0.68, Sugama et al., 1990). Fixed heterozygotes such as *ADA** at the distal end of chromosomes have also been observed by a number of other workers (Purdom et al., 1976; Cherfas and Truveller, 1978; Nagy and Csanyi, 1982; Thorgaard et al., 1983; Thompson and Scott, 1984; Seki and Taniguchi, 1989).

Purdom (1969), Nace et al. (1970) and Allendorf and Leary (1984) stated that the interruption of the second meiotic division (retention of second polar body) to induce diploid gynogenome progeny produces a proportion of heterozygotes depending on the rate of recombination between non sister chromatids during the first meiotic division. This will not be the case if gynogenesis is achieved by the suppression of the first mitotic division

if the first cell division is omitted (endomitosis) resulting progeny will be fully homozygous (degrees of inbreeding F = 1, Komen, 1990).

These and previous results show the limitations of using meiotic gynogenetics to produce completely homozygous inbred lines. Therefore, suppression of first cleavage at mitotic division of a zygote must be the more promising method for producing inbred lines in fish which will be homozygous at every gene locus (Streisinger et al. 1981; Chourrout 1984; Naruse et al. 1985; Purdom et al. 1985; Nagy 1987; Taniguchi et al. 1988; Komen et al. 1991a; Quillet et al. 1991 and the present study).

4.1.4.3 Outbred and inbred clonal lines:

Despite the limitation in the present study of only using two viable females, it is the first time clonal lines have been produced in an *Oreochromis* species. In two separate experiments, a sufficient number of outbred (OCL) and a few inbred (ICL) clones were produced. The use of the *ADA** marker locus enabled the families to be identified and confirmed no paternal inheritance in the ICL group and at they were homozygous and identical to maternal allele; all OCL progenies were heterozygous for the two differing homozygous parental alleles. Similarly, Han et al. (1991) also used isozyme markers (α*GPD-1**, *ME-1** and *PGM**) to identify the clonal lines in ayu, *Plecoglossus altivelis*.

Homozygous clones in zebra fish, *Brachydanio rerio*, were mostly free of recessive, deleterious, low penetrance alleles and were similar to starting population with respect to desirable characteristics (Streisinger et al. 1981). Similar results were demonstrated in medaka, *Oryzias latipes*, and ayu, *Plecoglossus altivelis*, respectively by Ijiri (1987) and

Han et al. (1991). Poor survival of normal progeny and presence of a high frequency of physiologically abnormal and deformed fry at hatching in both the ICL experiments suggests that the eggs from homozygous females *O. niloticus* might be highly sensitive to the shock treatments and thus resulted in the reduced survival, which is in contrast to the studies of Streisinger et al. (1981) and others. This phenomenon could not be verified because of a lack of sufficient numbers of reproducible mitotic females, therefore, further investigation in future is necessary.

In contrast the survival of the OCL involving eggs from the same females and fertilised with sperm of two homozygous mitotic gynogenetic males were highly satisfactory (25 - 40% survival at YSR) without any physiological abnormality and deformity in both the experiments. In this case the superior viability of outbred and hybrid nature of the cloned progeny compared to inbred type might be the effect of heterosis as has been outlined by Cherfas (1981) and Nagy (1987) cited by Komen (1990). More vigorous hybrid clones produced by crossing completely homozygous individuals were essentially free of lethal genes (Streisinger et al, 1981). Komen et al. (1991a) recently demonstrated the production of hybrid clones between homozygous females and XX mitotic gynogenetic males at the same time as he produced the gynogenetic clones of common carp (*Cyprinus carpio*). Komen et al. (1991b) observed that heterozygous type of F_1 hybrids showed an important reduction in variation for various morphological traits compared to homozygous clones. Although it was not attempted in the present study, a preliminary observation was that OCL of *O. niloticus* showed reduced phenotypic variations in various performance traits. Ihssen et al. (1990) stated that if a large part of the phenotypic variability is due to genetic variation among individuals, the phenotypic variability will be much smaller in an isogenic

line. They also explained that inbred lines will not be as useful, because their vitality is lower and they are not representative of the normal genotype of the species.

It appears that the OCL rather than ICL are preferred for the production of more viable and vigorous populations. More detailed studies using larger numbers of viable mitotic gynogenetic individuals will be necessary to identify females and males with the correct genotypes and phenotypes. Using such broodstock, a large number of F_1 hybrids could be produced. Hybrid or heterozygous clonal lines could be utilized for fixing novel and superior genes to be desirable for selective breeding and genetic improvement of tilapia and other important fish stock. It is expected that such vigorous clone will also be of great use as a pure "gene pool" for many genetic studies such as cell line and tissue culture, genetic fingerprinting, immunological, disease resistance, heritability and sex differentiation studies as well as developing breeding schemes based on the exploitation of heterosis.

A schematic model for the production of outbred and inbred clonal lines in *O. niloticus* based on the experimental protocol of the present study is presented in Fig. 4.1.4.3).

Production of clonal lines in fish is still one of the most difficult tasks because the viability and fertility of homozygous inbred strains produced by gynogenesis has been doubted (Kinghorn, 1983). In spite of developing homozygous gynogenetic diploids by the suppression of first mitotic division of fertilised eggs in a large number of fishes, until now cloning has been successful in only a few species such as *B. rerio* (Streisinger, et al. 1981); *O. latipes* (Naruse et al., 1985; Ijiri, 1987); *C. carpio* (Komen et al., 1991a); *P. altivelis* (Han et al., 1991) and *O. niloticus* (this report). Clonal lines will certainly be important

animals in fisheries and biological research, the potential of the commercial application of these lines in aquaculture and fisheries management is obvious but the recent nature of laboratory oriented work and lack of follow up trials makes this unlikely in near future.

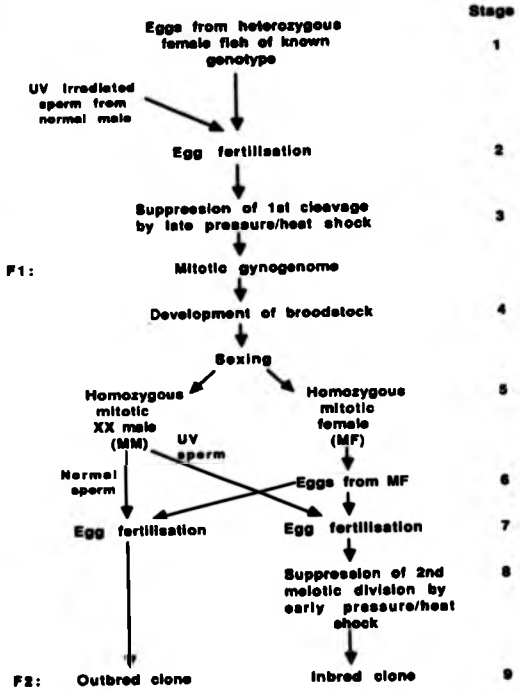


Figure 4.1.4.3. A schematic model for the production of outbred and inbred clonal lines in O. niloticus.

4.2 PHENOTYPIC VARIATIONS IN MEIOTIC AND MITOTIC GYNOGENETIC DIPLOIDS OF *O. NILOTICUS* L.

4.2.1 Introduction

Induced gynogenesis has been identified as a suitable technique for rapid production of highly inbred strains in fish. The inbred lines would offer great potential for the development of breeding strategies for the benefit of aquaculture (Nagy, 1987; Komen et al., 1991c). Gynogenesis achieved by inhibition of second meiotic division of eggs will always produce a proportion of heterozygotes depending on the rate of recombination between non sister chromatids during the first meiotic division (Purdom, 1969; Nace et al., 1970; Allendorf and Leary, 1984). This will not happen if gynogenesis is induced by the suppression of first mitotic cleavage and resulting progeny will be homozygous at every gene locus (see-Table 4.1.3.2). Therefore, in subsequent generation(s) the technique is of great advantage for producing pure lines with unique gene combinations.

Theoretically, it is believed that the phenotypic variation of particular trait(s) such as growth, various morphological parameters, gonadal and reproductive traits in the first generation of gynogenetic diploids will be increased with an increase in the degree of inbreeding (F value). Ultimately the mean value of a trait will be depressed in an inbred group with increased variance as the result of phenotypic expression of unmasked recessive alleles and reduced frequency of heterozygous loci (Falconer, 1981; Kincard, 1983; cited by Komen, et al. 1991c).

It has been seen recently (section 4.1.4.1) in the present fish (*O. niloticus*) that mitotic gynogenetics have a maximum value of degree of inbreeding (F = 1), therefore, it is expected that the level of phenotypic variation in particular trait(s) will be higher in this type of gynogenetic than that in meiotic gynogenetic diploids. Analysis of the phenotypic variation of a trait in gynogenetic diploids with a known degree of inbreeding can provide information for the estimation of heritability and genetic improvement of fish race for aquaculture (Taniguchi, 1990).

There are only three published reports on the study of phenotypic variation of various performance traits involving two type of gynogenetics (both meiotic and mitotic), one in ayu (Taniguchi et al., 1990) and two in common carp (Sumantadinata et al., 1990; Komen, et al. 1991c) but, none in *Oreochromis* spp.

The present study, therefore, aimed to investigate phenotypic variation of growth, meristic and various reproductive traits in meiotic and mitotic gynogenetic diploids compared to their control sibs of *O. niloticus* in order to ascertain the genetic characteristics of these fish. In the subsequent trials, genetic status and reproductive performance of homozygous gynogenetic males (recessive mutation in a sex determining gene) were also determined and discussed.

4.2.2 Materials and methods

4.2.2.1 Broodstock, breeding and production of meiotic and mitotic gynogenetic progeny:

The *O. niloticus* broodstock, their maintenance, feeding, stripping, egg fertilisation and incubation protocols used in this study, were as the relevant sections already described in Chapter 2. Eggs were collected from a heterozygous female and divided into three equal batches. The first and second batches of eggs were fertilised separately with UV irradiated sperm (section 2.5) and exposed respectively to optimal early and late shock treatments (pressure/heat, section 4.1.2.4). The third batch of eggs was fertilised with intact (un-irradiated) sperm of the same lot and these were used as a control.

4.2.2.2 Early fry rearing and on-growing:

Equal numbers of first feeding (7 - 8 days after hatching) meiotic, mitotic and control sibs were transferred from incubating jars into three 15-l plastic aquaria (section 2.1.2.1). During that time the fry were fed with *Artemia* nauplius. At 15 days a.h, meiotic (mean weight 0.0313±0.004 g), mitotic (mean weight 0.0298±0.004 g) and control (mean weight 0.0305±0.002) group of fry were each stocked into 20-l tanks at a density of 60 fry/tank for 3 weeks (section 2.1.1.2). At the end of the initial rearing period, at 5 weeks, meiotic, mitotic and control fry were restocked into three 500-l tanks at a density of 40 fish/tank and left for on-growing until 33 weeks in a controlled system (section 2.1.2.3). The early fry and on-growing fish were fed three and two times daily respectively with recommended dosage of various sizes of trout feeds (section 2.1.4).

At 2 weeks of age, initial estimation of growth (section 2.11) by weight and length (standard) were made on a random sub-sample by killing 10 larvae per treatment group. After that growth estimation of all the stocked fish (N = 40 per treatment) was carried out once every 4 weeks from 5 weeks to 29 weeks of age.

The experiment was terminated at 33 weeks, all the fish belonging to three treatment groups were manually sexed on the basis of the morphology of the urogenital papilla to determine the sex ratio (section 2.18). A total of 10 females from each group and 10 males from the mitotic and control group were randomly chosen, individually weighed and measured and finally killed to record the weight of ovaries and testes. The values for gonadosomatic index (GSI) was calculated as: individual gonad weight/individual body weight x 100

All the growth and gonadal data are given as means of each parameters estimated with their standard deviations. The coefficient of variation (C.V.) values were calculated as: Standard deviation values/mean x 100

4.2.2.3 Record of meristic characters:

A total of 20 fish/group were randomly selected from meiotic, mitotic and control groups at 33 weeks of age. Seven meristic characters dorsal fin rays, pectoral fin rays, anal fin rays, pelvic fin rays, lateral line scales and vertebrae were manually counted and recorded for individual fish. Data are presented as means of each meristic characters counted with their standard deviation. Coefficient of variation values were calculated as above.

4.2.2.4 Observation of ovulation response of meiotic, mitotic and control females:

As a part of the assessment of reproductive traits, ovulation or spawning response of 4 meiotic, 4 mitotic and 6 control females was observed in aquaria. Intensity of spawning was recorded by noting the intervals between two spawnings. Each female was observed over 3 and up to a maximum of 10 spawning periods. Then the mean value of the ovulation response of each female was calculated (total number of days continued regular spawning/total number of spawnings).

4.2.2.5 Crosses between control males vs meiotic, mitotic and control females:

A total of six crosses between control males and meiotic and control sib females were undertaken, some females were used more than once but in different crosses. Also 3 crosses involving 3 mitotic sib females and control males were also carried out. The viability of embryos at four development stages morula (MOR), pigmentation (PIG), hatching (HAT) and yolk sac resorption (YSR) were recorded (section 2.4). All values are presented as means of group wise crosses together with their standard deviation and coefficient of variation values.

4.2.2.6 Crosses between homozygous mitotic male vs meiotic, mitotic and control females:

To assess the reproductive performance of mitotic males in comparison to their controls, several crosses of mitotic males were made involving different females of meiotic, mitotic and control groups . Every time eggs were stripped from a female (meiotic or mitotic or control) they were divided into two equal batches. The first batch of eggs was fertilised with sperm from mitotic males and the second with sperm from the control male. A total

of six mitotic and 4 control males were selected for these crossbreeding experiments, some males were used more than once but in different crosses. Embryonic development of all these crosses was checked at HAT and YSR stages and recorded to determine the survival rate of normal fry, which are presented as means of all replicates with their standard errors.

4.2.1.7 Progeny testing for sex differentiation of immature young fish:

It was necessary in this study to check the sexes of at least a sub-sample of immature young fish derived from all the crosses involving mitotic males along with control males in an attempt to determine their genetic status. Sexing of at least 50 pooled progeny (5 - 7 weeks old) per cross was made by killing and examining the gonads of 1.5 - 2 g size young fish under microscope using the aceto-carmine squash technique (section 2.18) as described by Guerrero and Shelton (1974). The proportion of the sexes are given as a percentage.

4.2.2.8 Histology of meiotic, mitotic and control male testes:

The testes of matured meiotic, mitotic and control males were collected by killing the fish (3 fish/treatment group) and transferred to Bouin's fluid overnight before being finally fixed in 70% ethanol. Dehydrating, paraffin embedding and sectioning and staining of testes samples were made according to the recommended procedures (section 2.12). General histological examination was carried out under x63, x100, x250, x400 and x1000 (oil immersion) magnifications and photographed through an Orthomat Photomicroscope.

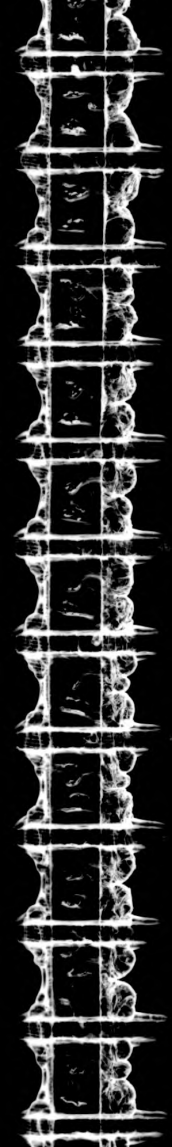
4.2.2.9 Cytological observation and estimating sperm concentration from meiotic, mitotic and control males:

Male donors were kept and reared one fish/tank in a series of 20-l tanks for 5 - 6 months before conducting this trial. A total of 3 meiotic, 5 mitotic and 5 control males were manually stripped and sperm was stored at 4 °C until further use (section 2.5). Sperm motility and concentration were checked and adjusted using a haemocytometer under the microscope (section 2.5).

4.2.2.10 Statistical analysis:

The genetic variation in growth rate, various meristic characters, gonadal and related morphological parameters and differences in sperm concentration of males between the treatment groups were analyzed by one way Analysis of Variance (ANOVA) . Significant results ($P<0.05$) or rejection of null hypothesis were further tested using Scheffe's Multiple Range Tests to identify significant differences between means using a Statgraphics Computer Package (Version 3.0).

Chi-square goodness-of-fit tests were performed to detect significant differences from expected 1:1 ratio between sexes in each cross-breeding experiment. Chi-square tests were also done using Statgraphics (Version 3.0).



4.2.3 Results

4.2.3.1 Variation in growth rate:

The mean and coefficient of variation (C.V.) values of growth in weight and length estimated for control, meiotic and mitotic gynogenome groups at successive ages from 2 to 29 weeks after hatching (a.h.) are presented in Table 4.2.3.1A and Table 4.2.3.1B. This experiment was not designed as a true growth study and periodical measurements of growth parameters such as weight and length were incidental to the collection of data on morphometric and reproductive traits.

Just after YSR (day 7 - 8 a.h.) until 15 days a.h. the early meiotic and mitotic gynogenome fry had a higher mortality rate but there were no significant differences ($P>0.05$) in weight or length compared to full sib controls. After that a marked variation in weight and length was observed between and among the groups at the start of exogenous feeding. At 5 weeks, the mitotic group first had a significantly ($P<0.001$) lower body weight (0.37±0.13SD g) and length (2.04±0.28SD cm) in comparison to meiotic (weight=0.54±0.1SD g; length=2.44±0.2SD) and control (weight=0.60±0.1SD g; length=2.55±0.2SD cm) groups. From 9 to 21 weeks of age, the growth rate in Cont..> Meio. > Mito. and values were significantly different ($P<0.001$) between the groups. During that time C.V.of body weight and length was lowest in control, intermediate in meiotic and greatest in mitotic gynogenome group. From the onset of sexual maturation (25 weeks) when no significant difference was observed between meiotic and mitotic groups until the end of the experiment (29 weeks). Although a significant deviation ($P<0.001$) was evident in growth rate between

Table 4.2.3.1A. Mean and coefficient of variation values of weight (g) for mixed sex control (Cont.), meiotic (Meio.) and mitotic (Mito.) gynogenetic groups at successive ages (from 2 to 29 weeks). All mean values from 5 to 29 weeks are based on 40 fish in each category except the 2 weeks of age, which was estimated from 10 fish per treatment. Significant differences of ANOVA and Scheffe,s Multiple Range Tests for comparisons among groups are shown by superscripts against each mean value in row.

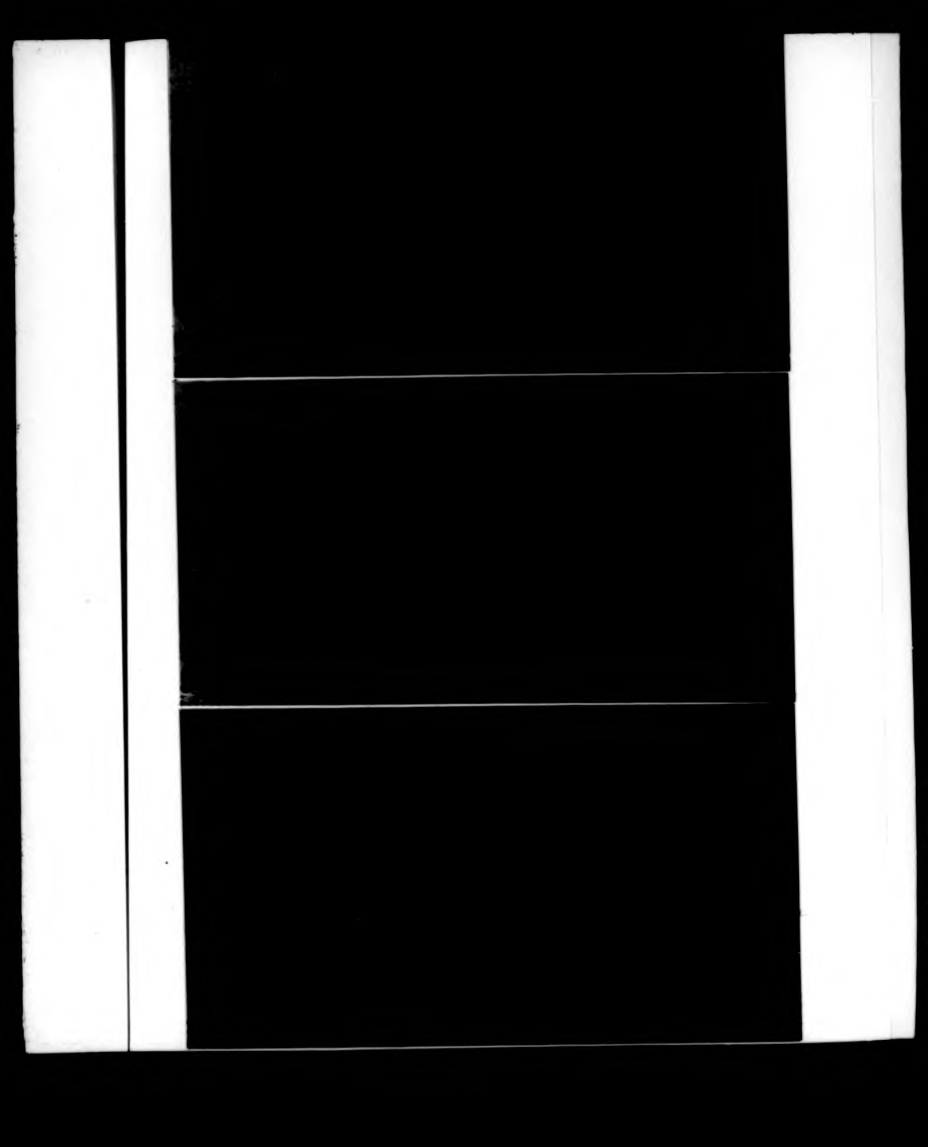
Age (Weeks)	Values	Cont.	Meio.	Mito.
2	Mean	0.0305[a]	0.0313[a]	0.0298[a]
	SD	0.002	0.004	0.004
	C.V.	6.56	12.78	13.42
5	Mean	0.60[a]	0.54[a]	0.37[b]
	SD	0.13	0.12	0.13
	C.V.	21.67	22.22	35.14
9	Mean	6.45[a]	4.96[b]	3.48[c]
	SD	0.40	1.16	1.68
	C.V.	6.20	23.39	48.27
13	Mean	24.00[a]	18.50[b]	10.91[c]
	SD	6.20	4.90	5.80
	C.V.	25.83	26.50	53.16
17	Mean	47.19[a]	36.16[b]	25.66[c]
	SD	12.00	9.66	13.70
	C.V.	25.43	26.71	53.39
21	Mean	89.91[a]	65.00[b]	47.91[c]
	SD	21.30	16.50	25.80
	C.V.	23.69	25.38	53.86
25	Mean	133.32[a]	94.15[b]	79.66[b]
	SD	40.40	25.60	37.90
	C.V.	30.30	27.19	47.58
29	Mean	177.64[a]	127.33[b]	113.27[b]
	SD	57.50	32.24	48.32
	C.V.	32.37	25.32	42.66

Table 4.2.3.1B. Mean and coefficient of variation values of length (cm) for mixed sex control (Cont.), meiotic and mitotic gynogenetic groups at successive ages (from 2 to 29 weeks). All mean values from 5 to 29 weeks are based on 40 fish in each category except the 2 weeks of age, which was estimated from 10 fish per treatment. Significant differences of ANOVA and Scheffe,s Multiple Range Tests for comparisons among groups are shown by superscripts against each mean value in row.

Age (Weeks)	Values	Cont.	Meio.	Mito.
2	Mean	1.24^{a}	1.25^{a}	1.23^{a}
	SD	0.035	0.04	0.04
	C.V.	2.82	3.20	3.25
5	Mean	2.55^{a}	2.44^{a}	2.04^{b}
	SD	0.21	0.23	0.28
	C.V.	8.24	9.43	13.72
9	Mean	5.55^{a}	5.05^{b}	4.15^{c}
	SD	0.30	0.40	0.80
	C.V.	5.41	7.92	19.28
13	Mean	8.55^{a}	7.78^{b}	6.19^{c}
	SD	0.67	0.70	1.2
	C.V.	7.81	9.00	19.39
17	Mean	10.91^{a}	9.80^{b}	8.12^{c}
	SD	0.87	0.84	3.44
	C.V.	7.97	8.57	42.36
21	Mean	13.07^{a}	11.97^{b}	10.17^{c}
	SD	1.30	1.26	2.40
	C.V.	9.95	10.53	23.60
25	Mean	15.16^{a}	13.55^{b}	11.98^{c}
	SD	1.50	1.10	2.30
	C.V.	9.89	8.12	19.20
29	Mean	16.55^{a}	15.13^{b}	13.66^{c}
	SD	1.74	1.23	2.42
	C.V.	10.27	8.13	17.71

Figure 4.2.3.2

Representative fish from diploid control, meiotic and mitotic gynogenetic groups at 33 weeks of age: showing size variations in body length.



control and the two gynogenetic groups but the ranking of C.V. changed as Meio.< Cont.< Mito. A comparative variation in size (length) of 33 weeks old Cont., Meio. and Mito. groups is shown in Fig. 4.2.3.2.

4.2.3.2 Variation in various meristic characters:

Table 4.2.3.2 shows the mean and coefficient of variation values of various meristic characters in control, meiotic and mitotic gynogenetic groups of *O. niloticus* at 33 weeks of age. The dorsal ray counts in the mitotic group showed a significantly ($P<0.001$) lower mean value (27.72±1.36SD) in comparison to the control group (28.75±0.54SD). The mean value of meiotic type (28.0±0.55SD) was not significantly different from either mitotic or control group. There was no significant deviation observed between meiotic and control groups for the mean number of pectoral fin rays and vertebrae but these were significantly smaller ($P<0.001$) in the mitotic type. The mean values for anal fin ray counts in meiotic (12.6±0.49SD) and mitotic (12.6±0.66SD) gynogenome groups were the same but significantly smaller ($P<0.001$) than the control (13.35±0.48SD) type. No significant deviation was found between and among the three groups for the mean values of lateral line scale counts, caudal and pelvic fin ray counts.

The coefficient of variation of all these meristic characters except for pelvic fin ray number among the groups showed a similar trend to the growth data in that C.V. was lowest in control, intermediate in meiotic and highest in mitotic gynogenetic group.

Table 4.2.3.2. Mean and coefficient variation values of various meristic characters of control, meiotic and mitotic gynogenetics of *O. niloticus* at 33 weeks of age. All mean values are based on 20 fish in each treatment. Significance differences of ANOVA and Multiple Range Tests are shown by superscripts against each mean values in column. Cont.= Control; Meio.= Meiotic gynogenetic; Mito.= Mitotic gynogenetic.

Characters	Treatment groups	Mean	SD	C.V.
Dorsal fin rays	Cont.	28.75^{a}	0.536	1.86
	Meio.	28.00^{ab}	0.548	1.96
	Mito.	27.72^{b}	1.364	4.92
Pectoral fin rays	Cont.	13.00^{a}	0.447	3.44
	Meio.	12.60^{a}	0.583	4.63
	Mito.	11.55^{b}	0.669	5.79
Anal fin rays	Cont.	13.35^{a}	0.477	3.57
	Meio.	12.60^{b}	0.490	3.89
	Mito.	12.60^{b}	0.663	5.26
Caudal fin rays	Cont.	16.25^{a}	0.433	2.66
	Meio.	16.15^{a}	0.357	2.21
	Mito.	15.95^{a}	0.384	2.41
Pelvic fin rays	Cont.	6.00	0.00	0.00
	Meio.	6.00	0.00	0.00
	Mito.	6.00	0.00	0.00
Lateral line scales	Cont.	30.65^{a}	0.792	2.58
	Meio.	30.90^{a}	0.831	2.68
	Mito.	31.20^{a}	1.288	4.13
Vertebrae	Cont.	29.85^{a}	0.357	1.196
	Meio.	30.05^{a}	0.497	1.652
	Mito.	28.35^{b}	1.590	5.61

4.2.3.3 Variation in reproductive traits:

a)Sex ratio data for control, meiotic and mitotic gynogenetic groups are given in Table 4.2.3.3A. At 33 weeks of age the sexes among the 40 meiotic gynogenetic progeny tested were 37 (92.5%) females and 3 (7.5%) were functional males. The observed frequencies of females in meiotic group was not different from expected 100% ratio. In contrast, among 40 mitotic gynogenomes, 20 (50%) were females, 1 (2.5%) had ovo/testes and 19 (47.5%) were functional males. Surprisingly the presence of a fairly high number of males in the mitotic progeny was probably the effect of a recessive sex determining gene due to inbreeding. Sexes in control group (N=40) derived from a conventional cross between normal male and female were 55% females and 45% males. The observed frequency of females and males in mitotic and control groups were not significantly different ($P>0.05$) from a 1:1 ratio according to the Chi-square test.

b) The mean and C.V. values of body weight, ovary weight and GSI in female (Table 4.2.3.3B) and body weight, testis weight and GSI in males (Table 4.2.3.3C) were estimated from control, meiotic and mitotic gynogenetic groups at 33 weeks of age. There was no significant difference in female body weight, ovary weight and GSI between the three groups but the ovaries of the mitotic group were usually of unequal size and were deformed. In males there were significant differences in mean body weight ($P<0.001$) and mean testis weight ($P<0.001$) but not GSI ($P>0.05$) between mitotic and control types. The C.V. values were largest in the mitotic type compared to those of meiotic (intermediate) and control (smallest) groups.

Table 4.2.3.3A.Observed frequencies of sexes in control (Cont.), meiotic (Meio.) and mitotic (Mito.) gynogenetic groups at 33 weeks of age. All values calculated in percentage are shown within parentheses.

Treatment group	Fish no.	Sexes (no.)		
		Female	Male	Bisexual
Cont.*	40	22(55)	18(45)	0
Meio.	40	37(92.5)	3(7.5)	0
Mito.*	40	20(50)	19(47.5)	1(2.5)

*Observed frequencies of females and males in control and mitotic gynogenetic groups were not significantly different (P>0.05) from expected 50% ratio according to the Chi-square test.

Table 4.2.3.3B. Mean and coefficient variation values of various morphological parameters of females from control, meiotic and mitotic gynogenetic groups at 33 weeks of age. All mean values are based on 10 fish in each treatment. Cont.= Control; Meio.= Meiotic gynogenetic; Mito.= Mitotic gynogenetic.

Parameters	Treatment group	Mean	SD	C.V.
Body weight (g)	Cont.	184.56	50.56	27.36
	Meio.	162.60	49.35	30.35
	Mito.	129.57	63.58	49.07
Ovary weight (g)	Cont.	2.46	0.80	32.52
	Meio.	2.51	2.37	90.41
	Mito.	1.66	2.28	137.34
GSI	Cont.	1.33	0.74	55.64
	Meio.	1.54	1.17	75.97
	Mito.	1.28	1.04	81.25

Table 4.2.3.3C. Mean and coefficient of variation values of various morphological parameters of males from control and mitotic gynogenetic groups at 33 weeks of age. All mean values are based on 10 fishes in each treatment. Significant differences of ANOVA and Multile Range Tests are shown by superscripts against each mean values in column. Cont.= Control; Mito.= Mitotic gynogenetic.

Parameters	Treatment group	Mean	SD	C.V.
Body weight (g)	Cont.	382.27[a]	36.6	9.58
	Mito.	178.93[b]	63.15	35.29
Testis weight (g)	Cont.	2.29[a]	0.90	39.30
	Mito.	0.62[b]	0.53	85.48
GSI	Cont.	0.60[a]	0.23	38.33
	Mito.	0.35[a]	0.48	137.14

Table 4.2.3.3D. Mean and coefficient of variation values for intensity of ovulation response of control, meiotic and mitotic gynogenetic females. Cont.= Control; Meio.= Meiotic gynogenetic; Mito.= Mitotic gynogenetic.

Parameter	Female group	Mean	SD	C.V.
Spawning intervals (days)	Cont. (N=6)	16.75	1.96	11.70
	Meio. (N=4)	12.69	2.39	18.83
	Mito. (N=4)	14.75	5.07	34.37

c) Female ovulation response experiment was carried out separately on individual females from each of the three treatment groups to observe the spawning response as it was suggested that inbreeding might have some effects on this particular trait of reproduction. No significant difference was evident for the mean values of spawning intervals among control (16.75±1.96SD day), meiotic (12.69±2.39SD day) and mitotic (14.75±5.07SD day) groups. But the C.V.values for spawning response ranked in Mito.> Meio.> Cont.(Table 4.2.3.3D).

d) Table 4.2.3.3E shows the mean and C.V. values of 4 embryonic development stages associated with different crosses between control, meiotic and mitotic females and control males of *O. niloticus*. The egg fertilisation rate was checked at MOR stage of embryonic development and did not show any significant deviation between and among the crosses. The viability of embryo survival at PIG, HAT and YSR stages did not show any significant differences between and among the crosses. Once again the C.V. at each development stage was different among the crosses lowest in Cont.x Cont., highest in the Mito.x Cont. and intermediate in Meio.x Cont.

4.2.3.4 Performance of homozygous gynogenetic males:

In histological sections, mitotic testes at 33 weeks af age contained normally developed and highly distinct tubules densely packed with spermatozoa (Fig. 4.2.3.5a). Control testes contained similar structures but the density of spermatozoa in the tubules was far less compared to the mitotic type (Fig. 4.2.3.5b). Testes of meiotic males were less well developed than those of mitotic and control counterparts most of the cysts beginning to fuse with a few spermatozoa (Fig. 4.2.3.5c).

Table 4.2.3.3E. Mean and coefficient of variation values of 4 embryonic development stages such as morula (MOR), pigmentation (PIG), hatching (HAT) and yolk sac resorption (YSR) of different crosses between control, meiotic gynogenetic, mitotic gynogenetic females and control males. Cont.= Control; Meio.= Meiotic gynogenetic; Mito= Mitotic gynogenetic.

Cross Female x Male	Embryo Development stage	Mean(%)	SD	C.V.
Cont. x Cont. (N=6)	MOR	88.0	10.00	11.36
	PIG	81.6	9.63	11.80
	HAT	68.7	10.30	15.00
	YSR	59.9	10.53	17.58
Meio. x Cont. (N=6)	MOR	84.7	16.86	19.90
	PIG	77.5	18.19	23.47
	HAT	54.6	20.18	36.96
	YSR	48.6	19.00	39.10
Mito. x Cont. (N=3)	MOR	71.4	17.31	24.24
	PIG	57.4	22.02	38.36
	HAT	44.5	28.66	64.40
	YSR	37.6	24.57	65.34

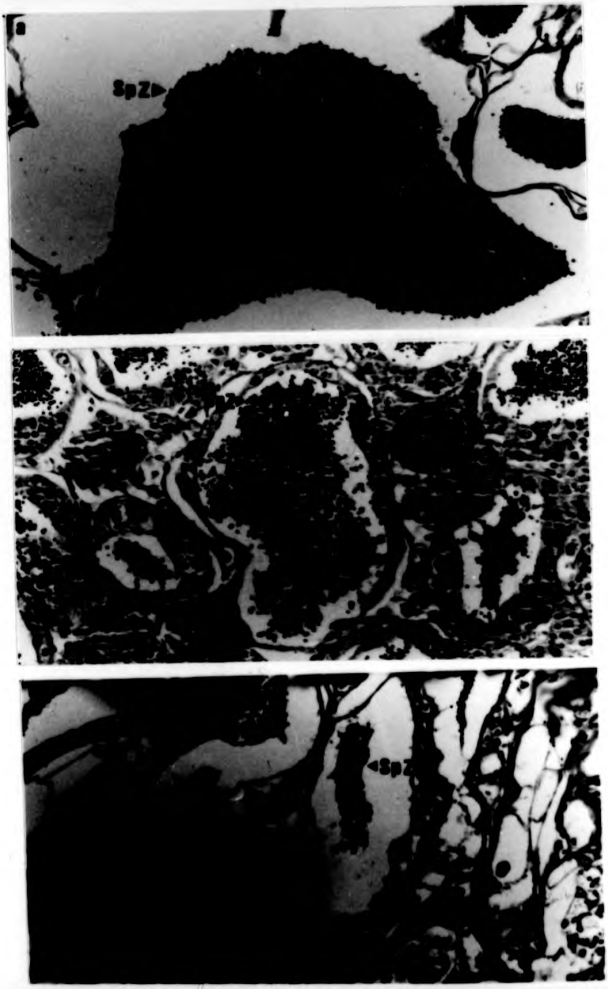
Figure 4.2.3.5

(a) Histological section of 33 weeks old mitotic gynogenetic testis (H & E, 77x).

(b) Histological section of 33 weeks old diploid control testis (H & E, 77x).

(c) Histological sections of 33 weeks old meiotic gynogenetic testis (H & E, 77x).

Legend SPZ = Spermatozoa


a
I
SpZb
◄Sp

Milt from mitotic males checked under the microscope displayed normal morphology and motility similar to those of control males. The mean concentration of undiluted sperm (Table 4.2.3.5A) was several times higher in mitotic ($3.12\pm1.0SE \times 10^9$, N=5) than control ($1.32\pm0.22SE \times 10^9$, N=5) and meiotic ($0.53\pm0.12SE \times 10^9$, N=3) males but these differences were not significant ($P>0.05$).

The data for mean survival rate of embryos at two development stages HAT and YSR and the sexes of progenies derived from different crosses between control, meiotic and mitotic females and mitotic males are presented in Table 4.2.3.5B. Eggs of control females fertilised with mitotic males showed better survival and less variability at HAT and YSR stages than those fertilised with control males. Crosses involving eggs from meiotic and mitotic females fertilized with control male produced fewer normal fry at both HAT and YSR compared to crosses with milt from mitotic males. But these difference were not significant.

The crosses between control, meiotic and mitotic females and mitotic males produced 100% female progenies. This result further suggested that all the homozygous gynogenetic males used in these crossbreeding trials belonged to XX genotypes. In contrast all the crosses involving control males produced the expected 1:1 sex ratio in the progenies.

Table 4.2.3.5A. Mean undiluted sperm concentration of control (Cont.), meiotic (Meio.) and mitotic (Mito.) gynogenetic males.

Serial number	Cont. (sperm ml^{-1})	Meio. (sperm ml^{-1})	Mito. (sperm ml^{-1})
1	0.88×10^9	0.30×10^9	3.53×10^9
2	1.27×10^9	0.80×10^9	6.59×10^9
3	2.16×10^9	0.48×10^9	4.10×10^9
4	1.53×10^9	-	0.61×10^9
5	0.78×10^9	-	0.75×10^9
Mean±SE	$1.32 \pm 0.22 \times 10^9$ (N=5)	$0.53 \pm 0.12 \times 10^9$ (N=3)	$3.12 \pm 1.00 \times 10^9$ (N=5)

Table 4.2.3.5B. Mean survival at hatching (HAT) and yolk sac resorption (YSR) and sexes of progenies of different crosses between control (Cont.), meiotic (Meio.), mitotic (Mito.) females and mitotic males. All values calculated in percentage are shown within parentheses.

Cross Fem.x Male	N	Survival(%±SE) HAT	YSR	Sex[g](no.) Female	Male
Cont.xMito.	10[a]	55.9± 8.4	50.8±8.8	50(100)	0
Cont.xCont.	4[b]	40.6±11.1	34.8±9.1	23(46)	27(54)
Meio.xMito.	6[a]	66.8± 6.9	58.4±7.0	50(100)	0
Meio.xCont.	3[c]	36.2± 6.1	32.6±5.2	26(52)	24(48)
Mito.xMito.	2[d]	32.5± 8.8	29.0±8.3	50(100)	0
Mito.xCont.	2[d]	24.3± 2.1	20.4±2.8	28(56)	22(44)

Experiments were conducted using [a]6, [b]4, [c]3 or [d]2 different males

[g]Observed frequencies of male and female sexes of progenies in crosses between Cont.x Cont., Meio.x Cont. and Mito.x Cont. were not significantly different (P>0.05) from expected 50% ratios according to Chi-square test.

4.2.4 Discussion

4.2.4.1 Variation in growth, meristic and reproductive traits in meiotic and mitotic gynogenetics:

In the present fish, the effect of a known degree of inbreeding on various phenotypic traits of two types of gynogenetics (meiotic and mitotic) in comparison to full sib controls was carefully observed. Among the performance traits, significantly reduced growth rate and an increase in the coefficient of variation in both inbred types compared with control counterparts apparent just a few weeks after stocking until maturity (21 weeks of age). The three full sibs experimental groups of fish were reared under the same density, same feeding and same environmental condition, therefore, these factors should not have any effect on the growth variation. The differences in food consumption due to less active feeding behaviour, reduced efficiency of physiological and metabolic processes are probably greatly influenced by inbreeding in such fish (Kaiser and Burns, 1981; Quillet et al., 1991).

A definite trend of C.V. was observed in six meristic characters Mito.> Meio.> Cont.>, which suggest the effects of reduced numbers of heterozygous loci due to different degrees of inbreeding. Similar variations for values of various meristic characters between two types of gynogenetics and the control group were reported by Taniguchi et al.(1990) and Summantadinata et al. (1990) respectively in ayu and common carp. Leary et al. (1984) observed that developmental instability and asymmetry of the meristic characters was an unfavourable effect of inbreeding in gynogenetic diploid rainbow trout.

In determining sex ratios of a gynogenetic population at 33 weeks of age of *O. niloticus* in the present study, the most surprising effect of inbreeding was presence of nearly 50% functional males in mitotic gynogenetic group; the meiotic group was nearly 100% female sex, the control cross produced the expected 1:1 ratio of sexes (see-Table 4.2.3.3A). A similar situation was also observed in several other gynogenetic experiments involving different females in the present fish. This frequency of mitotic males is consistent with the ratio of male (46.7%) vs female (53.2%) sexes observed in common carp mitotic populations by Komen (1990). In ayu, *P. altivelis*, Prof. N. Taniguchi (personal communication) also observed a fairly good proportion of males in mitotic gynogenetic progeny. In producing homozygous diploid clones of zebra fish by gynogenesis, Streisinger et al. (1981) found a considerable variation in the sex ratios among various clones and suggested that this was not consistent with either a simple female homogametic or female heterogametic system but possible autosomal sex-determining genes or environmental effects.

In the present species males are supposed to be heterogametic (XY) and females homogametic (XX) as suggested by Jalabert et al. (1974) and Hopkins (1979), therefore, both meiotic and mitotic progeny should be exclusively females (Penman et al., 1987a). This is the case in most meiotic gynogenetics produced in other fish species involving homogametic females (Stanley, 1976; Nagy et al., 1978; Chourrout and Quillet, 1982; Refstie et al., 1982; Chevassus, 1987). In a *O. niloticus* mitotic gynogenetic group the occurrence of certain proportion of males (designated as delta males) was reported by Mair (1988). Mair hypothesized that this mitotic gynogenetic male, therefore, corresponds to a sex reversed XX male and must be a result of a spontaneous sex reversal event governed

by a combination of rare autosomal sex modifying (to male) loci. Komen and Rickter (1990) stated that in common carp, the mitotic males are homozygous for a recessive mutation in a sex determining gene which induces a testis or (in complete penetrance) an intersex gonad in XX offspring.

Ovarian deformity and size abnormality in most of the mitotic females at 33 (a peak period of maturity) weeks of age compared to both meiotic and control group once more indicates the drastic effects of inbreeding on this most important trait of reproduction in the present fish. This observation is further consistent with the hypothesis of Dr. Komen (pers. communication) in common carp that many mature homozygous females show severe defects in their gonads and only a few (<10%) can successfully be reproduced. The depressed mean gonad weight and GSI with a higher coefficient of variation of these homozygous fish are the expression of retarded sexual development and might be due to a reduction in heterozygosity for the loci controlling gonadal growth, while the increasing C.V. is a result of the increasing level of inbreeding (Falconer, 1981).

The most striking effect of inbreeding depression was clearly observed in the final and ultimate stage of reproductive performance traits when females belonging to mitotic, meiotic and control groups were tested for crossbreeding with control males. The ovulation response of inbred female groups (both meiotic and mitotic) was reduced with increasing levels of inbreeding. Komen, (1990) found a similar phenomenon in common carp.

The present study revealed that the expansion of variation in growth, meristic and various reproductive traits in meiotic and mitotic gynogenetics was possibly the result of phenotypic

expression of unmasked homozygous recessive and deleterious genes as a result of the increasing degrees of homozygosity (F value). Therefore, it can be concluded with the hypothesis that the first generation of gynogenetics have limitations to use them directly for culture but they are potential and valuable especially the mitotic type as completely homozygous broodstock to produce second generation of clonal lines.

4.2.4.2 Genetic status and reproductive performance of homozygous gynogenetic males: The electrophoretic results at *ADA** and *EST-2** loci of six mitotic males indicated that they were all homozygous and had not inherited any paternal genomes (see- Table 4.1.3.3A). It was thus assumed that they should be all XX genotypic males. Further evidence of genetic status of mitotic males came when they all were tested for crossbreeding with control, meiotic and mitotic females. These crosses (N = 10) produced 100% female progenies which confirmed that they were all putative XX genotypic males resulting from a recessive mutation for a sex determining gene as suggested by Komen and Richter (1990).

The reproductive performance tests of mitotic males showed higher embryonic survivals at HAT and YSR stages (but not significant) in all crosses compared to control males. Komen (1990) observed significantly better yield of normal fry in control female vs mitotic male crosses.

The results of the present study has opened up a valuable new option. In that homozygous gynogenetic functional males could be used to produce heterozygous (hybrid) type of clones when crossed with completely homozygous females.

CHAPTER 5

BODY COLOUR INHERITANCE STUDIES IN RED TILAPIA STRAINS

BODY COLOUR INHERITANCE STUDIES IN RED TILAPIA STRAINS

5.1 INTRODUCTION

Red tilapias have become increasingly popular to fish farmers for their characteristic body colour, fast growth and high demand in the market. These strains are presently cultured commercially in many tropical and sub-tropical countries of the world such as Taiwan (Fitzgerald, 1979; Liao and Chang, 1983; Kuo, 1984); The Philippines (Radan, 1979; Galman et al., 1988); Thailand (Jarimopas, 1986; Huang et al. 1988); Malaysia (Siraj et al., 1988); Indonesia (Matricia et al., 1989); India (Rangaswami, 1988); Bangladesh (Akhteruzzaman et al. 1991); Guam (Fitzgerald, 1979); Greece (Anon, 1984b); Israel (Berger and Rothbard, 1987; Wohlfarth et al., 1990); Jamaica (cited by Wohlfarth et al., 1990); Brazil (P.C. Scott, pers. communication) and U.S.A (Sipe, 1977; Behrends et al., 1982; Behrends et al., 1988).

Commercially available red tilapia strains are mostly hybrids and the products of crossbreeding involving as many as four different species in which *O. mossambicus* and *O. niloticus* predominate (Brummet et al., 1988, McAndrew et al., 1988). There are still many doubts and confusions about the origin of these strains. The overall history of these mutant strains of tilapia is not properly documented.

The origin of Taiwanese red tilapia began in 1968, when several farmers discovered some red tilapia fry in southern Taiwan and brought them to the Taiwan Fisheries Research Institute, where their origin was checked and suspected to be an albino form of *O.*

mossambicus (Kuo, 1969; 1988). The stock was genetically inviable and was crossed with *O. niloticus*, thus the present red strain was developed as a hybrid (Liao and Chang, 1983; Kuo, 1988; Huang et al., 1988a). Initially, the founder hybrid strain did not produce a high frequency of red fry, but after several years of continued selection and hybridization trials using F_1 progeny, the proportion of red phenotypic fry was increased from 30% of the total population in 1969 to 80% in 1974 (Fitzgerald, 1979; Kuo, 1984; Kuo, 1988). Further genetic improvement of the Taiwanese red tilapia through crossbreeding experiments between red x red or with *O. aureus* and *O. niloticus* were also reported (Kuo and Tsay, 1984; 1985; 1986; 1987a; 1988).

The red tilapia strain in the Philippines was introduced from Singapore in 1978 and was reported to be a double hybrid between a cross of red female *O. mossambicus-hornorum* hybrid with Japanese strain of *O. niloticus* (Briggs, 1981, Galman et al., 1988).

The red *O. mossambicus* strain presently available in Israel was originally introduced from Singapore as an ornamental fish by aquarists. A sub-sample transferred to the Department of Zoology, Tel Aviv University, was identified as pure *O. mossabicus* stock, it was reproduced and distributed to other research stations (Pruginin, 1987; Wohlfarth et al., 1990).

Among U.S. strains of hybrid red tilapias, the first one reported derived from a crossbreeding involving *O. aureus*, *O. hornorum* and a red *O. mossambicus* strain (Sipe, 1979) and the second strain a cross between *O. hornorum* and red *O. mossambicus* (Behrends et al., 1982; Behrends and Smitherman, 1984). No more published information

is available on the Sipe's strain, but, the F_1 inbred progeny of the second strain was developed in Florida by a commercial fingerling producer who transferred some of them to the Tennessee Valley Authority and Auburn University for further research work in 1980 (Behrends et al., 1982), and are now available in many places of U.S.A.

Two strains of red tilapia were used for the present study (Red Egyptian and Thai red). The red Egyptian tilapia descended from a single male fish out of 100,000's of fry produced from an electrophoretically tested pure stock of Nile tilapia (Egyptian), *O. niloticus* (McAndrew and Majumdar, 1983; McAndrew et al., 1988), at the Institute of Aquaculture, University of Stirling, Scotland. The fish was subsequently grown to maturity and was mated with several normal coloured females to produce further generations of red progenies and preliminary information on the inheritance of this trait (McAndrew et al., 1988).

The origin of the Thai red strain is less certain and its uses were discussed for the first time in a workshop on "Tilapia Genetic Resources for Aquaculture" 1987 held in Bangkok, Thailand. Mr. Manob Tangtrongpiros, an official of the Thai Government, informed the meeting that red tilapia were found in a pond in northeastern Thailand, where *O. mossambicus* was introduced from Malaysia in 1949 (according to the report of Welcomme, 1981). Thus, this fish was assumed to be a hybrid between *O. mossambicus* and *O. niloticus*. According to Pullin (1988) electrophoretic analysis of Thai red tilapia samples from the Asian Institute of Technology (AIT) examined at the UPMSI laboratory, Philippines showed that both *O. mossambicus* and *O. niloticus* alleles were present. Recently, Dr. B.J. McAndrew and Mrs. P. Sodsook (Pers. communication) came to the same

conclusion after their electrophoretic analysis of the same Thai strain being held at the Institute of Aquaculture, University of Stirling.

Despite the commercial importance and development of several red tilapia strains in many regions of the world. One major problem of these mutant tilapia strains is that the majority of them do not breed true. Another problem is associated with the appearance of varying proportions of blotched types of fish in each generation, which are not as valuable to the consumers as the pure red individuals (McAndrew et al., 1988). It will be difficult to maintain or improve the quality of present stocks until the mode of body colour inheritance is well understood by hatchery workers and researchers.

Presently there are a few published reports available (for review see Wohlfarth et al., 1990 and section 1.3.4 of this thesis) on these aspects of red tilapia strains (Behrends et al., 1982; Scott et al., 1987; Huang et al., 1988a, 1988b; McAndrew et al., 1988; Mires, 1988; Tave et al., 1989). The inheritance of red body colour is complicated and unclear because of the hybrid nature of these fishes.

The present work, therefore, was aimed and designed to study in detail the Mendelian mode of red body colour inheritance as well as other related genetic mechanisms involved using gynogenesis in pure *O. niloticus* Egyptian red strain. An inheritance study was also conducted on Thai red tilapia, which is considered as one of the most promising commercial mutant tilapia strains in Southeast Asia.

5.2 MATERIALS AND METHODS

5.2.1 Source of parental strains

Red and wild type pure *O. niloticus* and Thai red tilapia used in this study were obtained from the Tilapia Reference Collection maintained at the Institute of Aquaculture, University of Stirling, Scotland (section 2.1.1). The broodstock of these strains were maintained in a recirculated water system (section 2.1.2) and were fed regularly with trout pellets (section 2.1.3).

5.2.2 Design of fish breeding experiments

Fish spawning, stripping and egg fertilisation protocols used in this study were as previously described (section 2.2). After stripping, the spent red or wild female was individually tagged (section 2.10) and returned to the broodstock tank and replaced with another rested sexually mature individual, thus guaranteeing frequent spawning.

5.2.2.1 Parental cross-breeding experiments:

a) Red x Red parental cross:

In red *O. niloticus* female x red *O. niloticus* male crosses, a total of 8 different females and 2 different males of red *O. niloticus* were used (Table 5.3.1A). In Thai red female x Thai red male crosses, a total of 6 different females and 2 different males were used (Table 5.3.3.A). Eggs stripped from a female were immediately fertilised with freshly collected milt from the selected male. No females in these crosses were used more than once and males (N=2) were used several times but with different females.

b) Red x Wild type Parental Cross:

In wild *O. niloticus* female x red *O. niloticus* male crosses, a total of 7 different wild type females and 9 different red males were mated (Table 5.3.1B).

In wild type *O. niloticus* female x Thai red male crosses, a total of 5 wild type females and 7 Thai red tilapia males were mated (Table 5.3.3B) . Eggs stripped from a female were immediately fertilised with freshly collected milt from the selected male (s). Some males and females in these crosses were used more than once but in different combinations.

5.2.2.2 Sib-cross-breeding experiments:

F_1 red female (red *O. niloticus* x wild *O. niloticus*) x F_1 red male (red *O. niloticus* x wild *O. niloticus*) crosses involved a total of 4 different females and 4 different males (Table 5.3.1C). In F_1 red female (Thai red tilapia x wild *O. niloticus*) x F_1 red male (Thai red tilapia x wild *O. niloticus*) crosses involved a total of 5 different females and 6 different males (Table 5.3.3C). Eggs stripped from a female were all either used for a single mating or divided into two or three equal batches (ca 300 - 400 eggs) and fertilised with selected male(s). Some females and males in these crosses were used more than once but in different combinations.

5.2.2.3 Back-cross experiments:

In wild type *O. niloticus* female x F_1 red (red *O. niloticus* x wild *O. niloticus*) male crosses, a total of 4 different females and 8 different males were used (Table 5.3.1D). In wild type *O. niloticus* female x F_1 red (Thai red tilapia x wild *O. niloticus*) male crosses, a total of 3 different females and 10 different males were used (Table 5.3.3D). Eggs stripped from

a female in most cases they were divided into 3 - 4 equal batches (ca 300 - 400 eggs) and fertilised with freshly collected milt of selected males. In these crosses, some males and females were mated more than once but in different crosses.

5.2.2.4 Gynogenetic reproduction experiments:

In total 6 different F_1 red (red *O. niloticus* x wild *O. niloticus*) heterozygous females used in these experiments were all derived from a single cross. Eggs stripped from a female fish were divided into two equal batches. The first batch of eggs was fertilised with UV irradiated (section 2.5) milt and exposed to either early heat (41°C for 3.5 min at 5 mins. after fertilisation) or pressure (8000 p.s.i. for 2 mins. at 9 mins. after fertilisation) shock treatment (section 2.3). The second batch of eggs was fertilised with intact normal milt from F_1 red male and was used as a control.

5.2.3 Egg incubation

Fertilised eggs of all normal crosses and treated and untreated (control) eggs of gynogenetic reproduction were identically incubated in a series of 750 ml round bottom plastic jars and survival rates of embryos at hatching and yolk sac resorption stages were recorded (section 2.4).

5.2.4 Fry rearing and on-growing

The early and advanced fry rearing and on-growing and maintenance were carried out according to the methods described earlier in section 3.2.6 and section 2,1.2. The fish were fed routinely with the recommended dosage of various sizes of trout feeds (section 2.1.4).

5.2.5 Scoring of progeny phenotypes

In the early stage (just after hatching or before yolk sac resorption) it was difficult to differentiate body colour pattern, therefore, phenotype scoring of most of the progeny sub-samples was done just one or two weeks after the first feeding stage with the help of a binocular dissecting microscope. The fish were anaesthetized (section 2.9) and killed if necessary for handling to score melanophores. In certain cases, if the body colour pattern was confused, progenies were left to grow for up to two months for rechecking of their phenotypes.

Progeny phenotypes in most of the crosses were basically categorised as "red" (including blotched type) and "wild type" (those were normally pigmented and completely different from those of the red phenotype). Only F_1 and gynogenetic progenies were differentiated between full red (approximately <10% body surface with melanophores) and blotched type (approximately >10% body surface with melanophores) as described by McAndrew et al. (1988), although both types together were termed as "red". To determine the observed ratio of colour segregation, the proportion of progeny phenotypes were calculated as (number of progeny of a given phenotype/total number of survivors) x 100.

5.2.6 Analysis of data

Observed body colour segregation ratio data of all parental (except red x red parental crosses), sib and back-crosses including gynogenetic reproduction were analyzed by Chi-square test of goodness-of-fit statistic (Statgraphics, Version 3.0).

5.3 RESULTS AND DISCUSSION

5.3.1 Inheritance of body colour in red *Oreochromis niloticus*

The results of crosses between red *O. niloticus* female and male parents (Fig. 5.3.1a) are presented in Table 5.3.1A. In total 8 different females were crossed with 2 randomly selected males in this experiment. The body colour segregation of progenies was 100% red in all these crosses (Fig. 5.3.1b). Therefore, it was presumed that the parental stock were either RR or Rr genotypes.

To understand more clearly the mechanism of inheritance of red body colour in this fish, other crosses involving wild type (normally pigmented) females and red males were made (Fig. 5.3.1c) and the results presented in Table 5.3.1B. Among 10 crosses, progenies in 7 crosses segregated into red and wild type individuals. The proportion of red (including blotched type) progenies in these seven crosses varied between 43.3% and 55.9%, of which only number 7 significantly deviated ($P<0.05$) from expected 1:1 ratio. This indicated that all red males involved in these seven crosses were presumably heterozygotes (Rr) and when they were crossed with wild (rr) females gave a phenotypic ratio of red:wild close to 1:1. In the remaining 3 crosses (cross no. 4, 5, 6) all progenies segregated into 100% red and 0% wild type (Fig. 5.3.1d).

Frequency estimates of the blotched pattern in the red phenotype were also attempted in F1 progenies derived from either RR x rr or Rr x rr parental crosses in this study (Table 5.3.1B). McAndrew et al. (1988) classified fish with <10% of body surface with

Table 5.3.1A. Results of parental crosses between red *O. niloticus* females and males.

Cross no.	Parent's tag		Progeny no.	Progeny phenotype		Proportion of red (%)
	Red female	Red male		Red (no.)	Wild (no.)	
1	0604	0606	123	123	0	100
2	0605	0606	130	130	0	100
3	0610	0606	80	80	0	100
4	0611	0606	178	178	0	100
5	0612	0606	125	125	0	100
6	0619	0618	150	150	0	100
7	0620	0618	155	155	0	100
8	0621	0618	150	150	0	100

Figure 5.3.1

(a) Representative female and male red *O. niloticus* used for red x red parental cross.

(b) Phenotypes of F_1 progeny derived from red x red parental cross of *O. niloticus*.

Table 5.3.1B. Body colour segregation in F_1 progenies derived from parental crosses between wild type *O. niloticus* females and red *O. niloticus* males.

Cross no	Parent.s tag		Pro-geny no.	F_1 Progeny phenotype			Propor* tion of red (%)	Chi-square
	Wild female (Presumed genotype)	Red Male		Red		Wild type (no).		
				Full (no.)	Blotch (no.)			
1	0924 (rr)	1397 (Rr)	343	92	91	160	53.4	1.542
2	0924 (rr)	1361 (Rr)	136	41	35	60	55.9	1.882
3	0511 (rr)	0468 (Rr)	144	3	70	71	50.9	0.028
4	0512 (rr)	0516 (RR)	96	10	86	0	100	0.000
5	0474 (rr)	0473 (RR)	78	7	71	0	100	0.000
6	0415 (rr)	0516 (RR)	72	67	3	0	100	0.000
7	0415 (rr)	0515 (Rr)	80	20	10	50	37.5	5.000*
8	0425 (rr)	0478 (Rr)	89	23	16	50	43.8	1.360
9	0415 (rr)	0414 (Rr)	92	13	35	44	52.2	0.677
10	0477 (rr)	0476 (Rr)	104	14	31	59	43.3	1.188

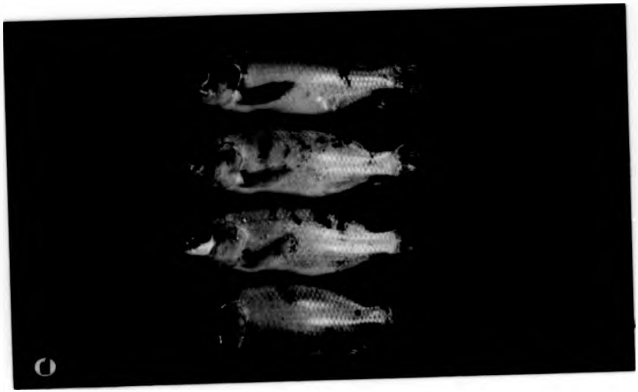
*Expected proportion of red in crosses RR x rr = 100% and crosses Rr x rr = 50%.
*$P<0.05$

Figure 5.3.1

(c) Representative wild type female and red *O. niloticus* male used for red x wild parental cross.

(d) Phenotype of F_1 progeny derived from red x wild parental cross of *O. niloticus*.

B



C

melanophores as full red and those with >10% were designated as blotched. Some crosses (cross no. 4 and 5) involving both homozygous red male (RR) and wild female (rr) produced a higher percentage of blotch pattern (Rr) in F_1 progenies (Fig. 5.3.1d) compared to most of the crosses between heterozygous red males (Rr) and homozygous wild females (rr). But this trend was not consistent with the cross no. 3 and 6. Frequency of blotched phenotypes in these two crosses suggest that blotch is acting as a single gene but it is difficult to identify, "B" might be dominant but epistatic to "R". Therefore, it was presumed that in cross no. 3 nearly all red blotch progeny genotypes were either RrBB or RrBb. In contrast, nearly all red unblotched progeny in cross no. 6 presumably belonged to Rrbb genotype. McAndrew et al. (1988) stated that blotched phenotype is hypostatic to the red gene and can only be expressed in its presence. Thus, it appears that this gene is totally suppressed in the normal coloured (wild type) fish or its expression is different in the absence of the red gene.

To determine and confirm more precisely the mode of inheritance of the dominant red gene (R) action over recessive wild type (r) a series of breeding trials including F_1 sib cross (Table 5.3.1C and Fig. 5.3.1e) and back-cross (Table 5.3.1D and Fig. 5.3.1g) were carried out. The segregation of progeny phenotypes in sib and back-crosses are shown in Fig. 5.3.1f and Fig. 5.3.1h respectively. The proportion of red progenies in 10 crosses among F_1 heterozygous full-sibs (red *O. niloticus* x wild *O. niloticus*) red progenies ranged between 68% and 75.6% and wild progenies between 24.4% and 32%. The data analysis of these observed phenotypic ratios with Chi-square goodness-of-fit test revealed that they were not significantly different ($P<0.05$) from expected 3 red:1 wild ratio. In 10 different back-

Table 5.3.1C. Body colour segregation in F_2 progenies derived from sib crosses between F_1(red *O. niloticus* x wild *O. niloticus*) red females and F_1(red *O. niloticus* x wild *O. niloticus*) red males. Observed proportion of F_2 progeny phenotypes are shown in parentheses.

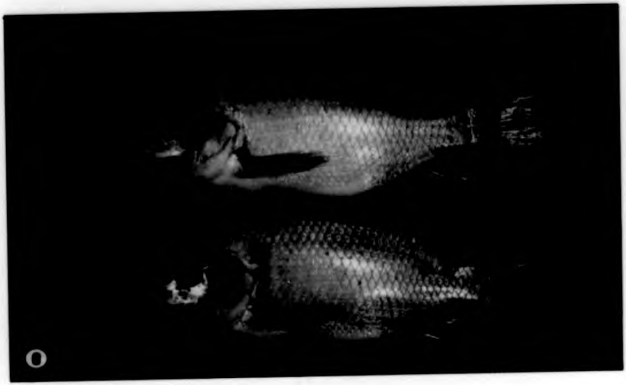
Cross no	F_1 parent's tag			F_2 progeny phenotype		
	Red female (Rr)	Red male (Rr)	Progeny no.	Red no.(%)	Wild no.(%)	Chi-square
1	0745	0743	128	87(68.0)	41(32.0)	3.375
2	0745	0744	162	122(75.3)	40(24.7)	0.006
3	0746	0749	109	80(73.4)	29(26.6)	0.149
4	0747	0749	180	132(73.3)	48(26.7)	0.267
5	0748	0749	114	86(75.4)	28(24.6)	0.012
6	0746	0750	172	130(75.6)	42(24.4)	0.031
7	0748	0750	198	149(75.3)	49(24.7)	0.006
8	0747	0750	187	137(73.3)	50(26.7)	0.271
9	0746	0751	183	133(72.7)	50(27.3)	0.526
10	0747	0751	110	78(70.9)	32(29.1)	0.982

Expected proportion of red in crosses Rr x Rr = 75%

Figure 5.3.1

(e) F_1 (red x wild *O. niloticus*) red female and male used for sib-cross.

(f) Body colour segregation in F_2 progeny derived from sib-cross between F_1 (red x wild *O. niloticus*) red female and male.



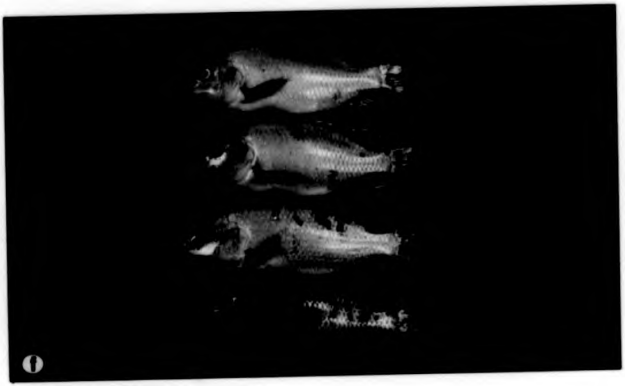


Table 5.3.1D. Body colour segregation in progenies derived from back-crosses between recessive wild (*O. niloticus*) females and F_1(red *O. niloticus* x wild *O. niloticus*) heterozygous males. Observed proportion of progeny phenotypes is shown in parenthesis.

Cross no.	Parent's tag			Progeny phenotype		
	Wild female (rr)	F_1 red male (Rr)	Progeny no.	Red no.(%)	Wild no.(%)	Chi-square
1	0506	1390	213	107(50.2)	106(49.8)	0.004
2	0502	0706	240	119(49.6)	121(50.4)	0.016
3	0502	0367	168	85(50.6)	83(49.4)	0.024
4	0502	1390	758	368(48.5)	390(51.5)	0.638
5	0580	1394	291	155(53.3)	136(46.7)	1.240
6	0580	0370	301	161(53.5)	140(46.5)	1.465
7	0580	1392	261	136(52.1)	125(47.9)	0.464
8	0580	1390	309	134(43.4)	175(56.6)	5.440*
9	0580	0752	377	181(48.0)	196(52.0)	0.597
10	0569	0747	434	211(48.6)	223(51.4)	0.332

Expected proportion of red in crosses Rr x rr = 50%
*P<0.05

Figure 5.3.1

(g) Showing recessive wild *O. niloticus* female and F_1 (red x wild *O. niloticus*) red male used for back-cross.

(h) Body colour segregation in progeny derived from back-cross between recessive wild *O. niloticus* female and F_1 (red x wild *O. niloticus*) red male.

crosses between 4 wild recessive females and 7 F_1 red males , progenies were segregated into red (between 43.4% and 53.5%) and wild type (between 46.5% and 56.6%) and the only significant deviation ($P<0.05$) from expected 1:1 ratio was in cross no. 8.

The present results of all parental, sib and back-crosses, therefore, show that red body colour in *O. niloticus* is acting as a dominant in the presence of wild type. In contrast, all wild type were found to be pure homozygous recessives (rr). This dominant red trait controlled by single autosomal gene in the same strain (McAndrew et al., 1988) and some other tilapia strains (Behrends et al., 1982; Behrends and Smitherman, 1984; Avtalion and Reich, 1989; Hilsdorf, 1990; Huang et al., 1988b). On the other hand, blond body colour of *O. niloticus* (Scott et al. 1987, McAndrew et al., 1988; Mires, 1988) and red or gold colour of *O. mossambicus* (Pruginin, 1987; Tave et al., 1989; Wohlfarth et al., 1990) were explained as simple Mendelian recessive. The majority of colour variants in fish are recessive traits in their mode of inheritance, particularly those reported in carp (Kirpichnikov, 1981). The incomplete dominance was also observed in Taiwanese red tilapia by Huang et al. (1988a) and Wohlfarth et al. (1990).

The dominant nature of the red body colour in this strain of *O. niloticus* has important implications for genetic research and in the development of pure breeding population. The existing broodstock population are a mixture of both homozygous (22.2%) RR individuals that breed true, and heterozygous (77.8%) Rr individuals, that will not. This problem arose because the single ancestor fish was reported to be a heterozygote (Rr, a spontaneous mutant) and successive generations were mostly or entirely derived from the crosses between red x wild type at the Institute of Aquaculture, University of Stirling (McAndrew

et al., 1988). Thus, the founder stock a mixture of homozygous (RR) and heterozygous (Rr) progenies as no attempts at progeny testing were made.

In the presence of such a high frequency of heterozygotes in the parental stock, a true-breeding population can not be developed until a viable method to identify the various genotypes is available. In the mixed genetic population, when they are bred, one will get the segregation of wild types and blotched individuals. Completely red fish are much more acceptable to the consumers than the blotch type. If all red mutant brooders are desired to be true breeding in a population, they should obviously be fixed as homozygous at the "R" allele, therefore the undesirable "r" allele must be culled in some way. In this case the Mendelian test-cross technique to differentiate 'RR' homozygous broodstock and to maintain their selective breeding for one or two generations would be a probable solution. Once it is done, this red *O. niloticus* can widely be used for commercial aquaculture.

5.3.2 Inheritance of body colour in gynogenetic progenies derived from F_1 heterozygous red females

The results of six gynogenetic trials involving six different F_1 (red *O. niloticus* x wild *O. niloticus*) red females are presented in Table 5.3.2. In this type of gynogenetic reproduction involving heterozygous (Rr) red female, according to Nace et al. (1970), Thorgaard et al. (1983) and Streisinger et al (1986) 1) no heterozygotes are expected if no crossing over takes place between the gene and its centromere, the frequencies of both mutant (RR) and wild type (rr) homozygous are equal; 2) the varying proportion of heterozygotes (Rr) up to 100% are expected depending on the amount of crossing over between the respective gene and its centromere.

Among six independent trials, progenies were segregated into red and wild type phenotypes except Expt. no. 3, where all the progenies were red but intensly blotched, Y = 1.0, they were presumably 100% recombinants (Rr) for mutant and wild type alleles (proportion of heterozygotes were calculated as the equation described by Nace et al., 1970; see Table 5.3.2). In Expt. 1 and 2, progenies were more or less equally segregated into red and wild type and the observed frequencies of body colour phenotypes were not significantly different from expected 1 red: 1 wild ratios, although the frequencies of their calculated "Y" value ranged between 0.023 and 0.040 these were negligible and very close to 0. This suggests that in such cases no recombination took place between the gene and its centromere, thus all red phenotypic progenies were presumably homozygous for the "R" alleles and all wild type progenies were homozygous for the "r" alleles. The progenies in the three other trials (Expt. 4, 5 and 6) segregated into red, blotched and wild type with varying frequencies of hetrozygotes (Y) ranged from 0.50 to 0.625 due to varying recombination rates between gene and its centromere during first meiosis. The observed phenotypic frequencies of six gynogenetic progenies were not significantly different from expected 1:1 ratio for trials 1 and 2; 1:0 ratio for trial 3; 3:1 ratio for trials 4, 5, 6. In contrast, the observed segregation of body colour pattern in progenies derived from all control crosses were not significantly different from expected ratio of 3 red: 1 wild type, though the results are not included in Table 5.3.2.

Although the present experiment was aimed to observe the mechanism of body colour inheritance in gynogenetic progenies derived from heterozygous mutant female. It suggest that gynogenesis could be used as a tool to differentiate progenies between red homozygotes and heterozygotes.

Table 5.3.2. Distribution of body colour pattern and frequency of heterozygotes in meiotic gynogenetic progenies derived from six red F_1(Red *O. niloticus* x wild *O. niloticus*) heterozygous females.

Expt. no.	Female tag no. (Presumed geno type)	Progeny no.	Progeny phenotype: Red: Full (no.)	Red: Blotch (no.)	Wild (no.)	Expected % wild	Proportion of heterozygote (Y)	Chi-square
1	0757 (Rr)	43	22	0	21	50	0.023	0.023
2	0759 (Rr)	25	13	0	12	50	0.040	0.040
3	0761 (Rr)	9	0	9	0	0	1.000	0.000
4	0762 (Rr)	16	3	9	4	25	0.500	0.000
5	0760 (Rr)	26	8	12	6	25	0.538	1.867
6	0763 (Rr)	32	8	18	6	25	0.625	0.667

Note: 1. Observed frequencies of body colour pattern in progenies derived from all control crosses related to the above experiments were not significantly deviated from expected Mendelian segregation ratio of 3 red: 1 wild type.
2. Proportion of heterozygotes (Y) = Proportion of dominants (Red) minus recessives (wild), which were calculated as the equation described by Nace et al. (1970).

5.3.3 Inheritance of body colour in Thai red tilapia

Table 5.3.3A exhibits the data of 6 different crosses between Thai red tilapia females and males. Six different females were mated with 2 different males in this experiment (Fig.5.3.3a). In these crosses all the progenies produced were 100% red (Fig. 5.3.3b)), thus presumed genotypes of both the females and males were either "RR" (homozygotes) or "Rr" (heterozygotes) or a combination of both the genotypes.

A further investigation using wild type *O. niloticus* females and Thai red tilapia males was undertaken (Fig. 5.3.3c) and the results of these crosses are shown in Table 5.3.3B. In total 8 different crosses were carried out between 5 females and 6 males, the progenies segregated into 100% red and 0% wild type (Fig. 5.3.3d) in 7 of these crosses indicating that 5 red males participating in these mating were presumably homozygotes (RR). In the remaining cross (cross no. 6), the progeny segregated into 41.8% red and 58.2% wild, the male was presumably a "Rr" heterozygote and progeny ratio was not significantly different from expected 1:1 ratio. A total of 5 different wild type females used in these crossbreeding trials were found to be all recessive homozygotes (rr). An attempt was made in this experiment to differentiate between full red (<10% body surface with melanophore) and blotched (>10% body surface with melanophore) type progenies as described by McAndrew et al. (1988). But no clear cut pattern of segregation of blotching was observed in these crosses. Although red colour is found to be a completely dominant phenotypic trait and most of the brooders (83.3%) of the Thai red tilapia are homozygous at the dominant "R" allele (RR) a certain percentage of them (16.7%) are heterozygous at the "R" allele (Rr),

Table 5.3.3A. Results of parental crosses between Thai red tilapia females and males.

Cross no.	Parent's tag		Progeny no.	Progeny phenotype		Proportion of red (%)
	Red female	Red male		Red (no.)	Wild (no.)	
1	0602	0603	150	150	0	100
2	0604	0603	106	106	0	100
3	0605	0603	185	185	0	100
4	0621	0624	305	305	0	100
5	0622	0624	250	250	0	100
6	0623	0624	235	235	0	100

Figure 5.3.3

(a) Representative female and male red Thai tilapia used for red x red parental cross.

(b) Phenotype of progeny derived from red x red parental cross of Thai tilapia strain.

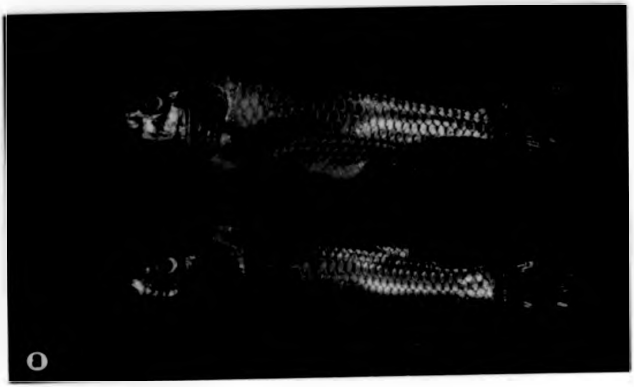


Table 5.3.3B. Body colour segregation in F_1 progenies derived from parental crosses between wild type *O. niloticus* females and Thai red tilapia males.

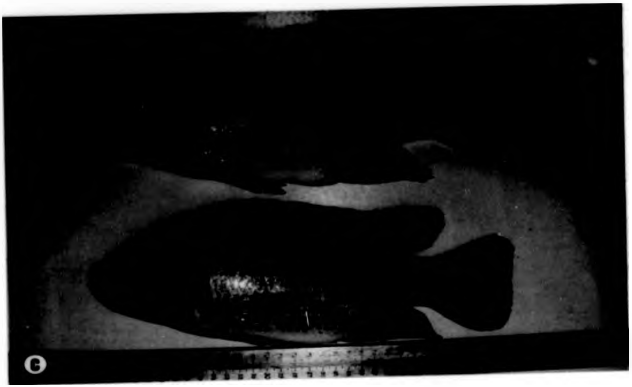
Cross no	Parent's tag: Wild female (Presumed genotype)	Parent's tag: Red Male (Presumed genotype)	Progeny no.	Progeny phenotype: Red: Full (no.)	Progeny phenotype: Red: Blotch (no.)	Progeny phenotype: Wild type (no.)	Proportion of red (%)*	Chi-square
1	1320 (rr)	0931 (RR)	49	20	29	0	100	0.000
2	0977 (rr)	0978 (RR)	154	52	102	0	100	0.000
3	0425 (rr)	0411 (RR)	337	23	314	0	100	0.000
4	0930 (rr)	0416 (RR)	129	71	58	0	100	0.000
5	0425 (rr)	0416 (RR)	143	89	54	0	100	0.000
6	0472 (rr)	0471 (Rr)	146	0	61	85	41.8	3.945
7	0472 (rr)	0411 (RR)	116	50	66	0	100	0.000
8	0425 (rr)	0547 (RR)	90	78	12	0	100	0.000

*Expected proportion of red in crosses RR x rr = 100% and Rr x rr = 50%.

Figure 5.3.3

(c) Representative female wild type *O. niloticus* and male Thai red tilapia used for red x wild parental cross.

(d) Phenotype of F_1 progeny derived from red Thai tilapia x wild *O. niloticus* parental cross.



In the present study, the presumed mode of red body colour inheritance in Thai red tilapia was finally confirmed as Mendelian by the pattern of breeding tests involving F_1 sibs mated *inter se* (Table 5.3.3C) and back-crossed with recessive wild type females (Table 5.3.3D). A total of 9 crosses were undertaken between F_1 red sibs (Thai red tilapia x wild *O. niloticus*, Fig. 5.3.3e) and F_2 progenies in all these crosses were segregated into red and wild phenotypes (Fig. 5.3.3f). The observed proportion of red progenies ranged between 67% and 76% and wild progenies between 22.6% and 33.0%. The observed segregation of the progeny phenotypes was significantly different ($P<0.001$) only in cross 6 from the expected 3 red:1 wild ratio.

Table 5.3.3D summarizes the results of 11 back-crosses between recessive wild *O. niloticus* females and F_1 red (Thai red tilapia x wild *O. niloticus*) males (Fig. 5.3.3g). In all these crosses progenies were segregated into red and wild type individuals (Fig. 5.3.3h). The proportion of red phenotypic individuals in 11 crosses ranged between 37.4% and 52.5%. The wild type ranged between 47.5% and 62.6%. Only in cross no. 4, was the distribution of colour pattern significantly different ($P<0.05$) from expected 1:1 ratio.

In this study, the results of all parental, sib and back-crosses demonstrate and suggest that red body colour in Thai red tilapia strain is a simple autosomal dominant trait controlled by a single "R" gene with two alleles, "R" dominant for red colour over "r" recessive for wild type. A similar mode of body colour inheritance in the same strain has been explained by Hilsdorf (1990). But the present results are in contrast to those of other studies previously conducted in Taiwanese red tilapia (Huang et al., 1988a; Wohlfarth et al., 1988)

Table 5.3.3.C. Body colour segregation in F_2 progenies derived from sib-crosses between F_1(Thai red tilapia x wild *O. niloticus*) red females and F_1(Thai red tilapia x wild *O. niloticus*) red males. Observed proportion of F_2 progeny phenotypes is shown in parentheses.

	F_1 parent's tag			F_2 progeny phenotype		
Cross no.	Red female (Rr)	Red male (Rr)	Progeny no.	Red no.(%)	Wild no.(%)	Chi-square
1	0701	0705	243	188(77.4)	55(22.6)	0.726
2	0702	0706	132	97(73.5)	35(26.5)	0.162
3	0703	0705	164	125(76.0)	39(24.0)	0.053
4	0704	0707	325	247(76.0)	78(24.0)	0.053
5	0712	0709	416	310(74.5)	106(25.5)	0.013
6	0712	0711	336	225(67.0)	111(33.0)	11.571***
7	0716	0707	254	192(75.6)	62(24.4)	0.047
8	0716	0709	100	77(77.0)	23(23.0)	0.213
9	0716	0714	201	151(75.1)	50(24.9)	0.001

Expected proportion of red in crosses Rr x Rr = 75%;
*P<0.05 ; **P<0.01; ***P<0.001

Figure 5.3.3

(e) F_1 (red Thai tilapia x wild *O. niloticus*) red female and red male used for sib-cross.

(f) Body colour segregation in F_2 progeny derived from sib-cross between F_1 (red Thai tilapia x wild *O. niloticus*) red female and red male.

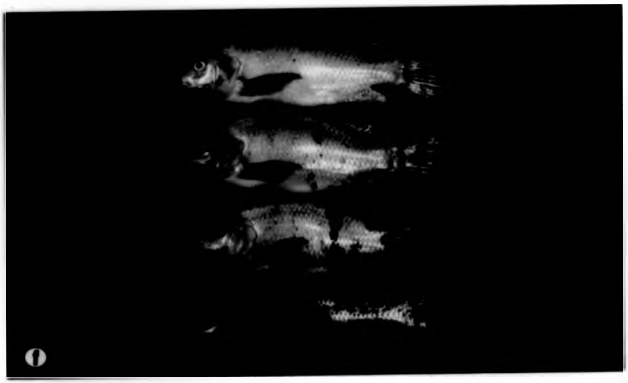


Table 5.3.3D. Body colour segregation in progenies derived from back-crosses between recessive wild (*O. niloticus*) females and F_1(Thai red tilapia x wild *O. niloticus*) heterozygous red males. Observed proportion of progeny phenotypes is shown in parentheses.

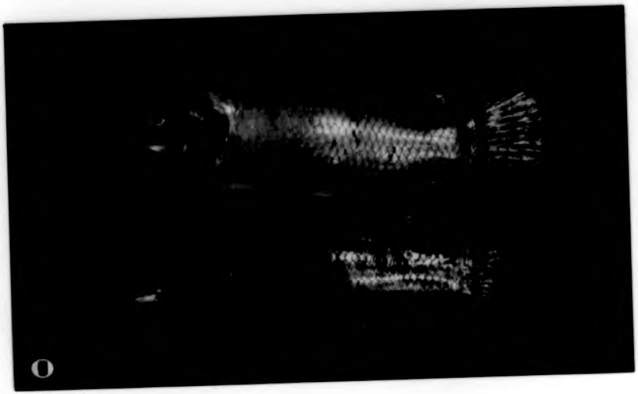
Cross no.	Parent's tag		Progeny no.	Progeny phenotype		Chi-square
	Wild female (rr)	Red male (Rr)		Red no.(%)	Wild no.(%)	
1	0506	0397	280	124(44.3)	156(55.7)	3.657
2	0596	0390	144	67(46.5)	77(53.5)	0.694
3	0596	0391	141	74(52.5)	67(47.5)	0.347
4	0596	0392	107	40(37.4)	67(62.6)	6.813*
5	0930	0707	308	157(51.0)	151(49.0)	0.117
6	0930	0708	457	210(46.0)	247(54.0)	2.996
7	0930	0709	267	118(44.2)	149(55.8)	3.599
8	0930	0710	345	164(47.5)	181(52.5)	0.838
9	0715	0707	126	61(48.4)	65(51.6)	0.127
10	0715	0713	235	105(44.7)	130(55.3)	2.659
11	0715	0714	201	98(48.8)	103(51.2)	0.124

Expected proportion of red in Rr x rr crosses = 50%;
*P<0.05

Figure 5.3.3

(g) Showing recessive wild *O. niloticus* female and F_1 (red Thai tilapia x wild *O. niloticus*) red male used for back-cross.

(h) Body colour segregation in progeny derived from back-cross between recessive wild *O. niloticus* female and F_1 (red Thai tilapia x wild *O. niloticus*) red male.



and Philippine red tilapia (Galman, 1987). These workers concluded that mode of red body colour inheritance in the Taiwanese starins as a single gene with incomplete dominance.

These results suggest that the red colour in both strains is controlled by a single dominant "R" gene. Both red strains contains certain proportions of heterozygotes (Rr) in the existing broodstock. In order to produce pure breeding strains it will be important to identify heterozygotes so as the undesirable "r" gene can be removed from the population. At present it appears that the Mendelian back-cross is the only viable means of identifying such fish. Blotched phenotype can not be eliminated unless a clear cut description of the level of blotching can be determined as a viable trait. This will require more detailed study. The possible option is to reduce the level of blotching from a population by isolating red unblotched homozygotes (RRbb) and use them as broodstock. Once it is done, production of all pure red progenies of the two mutant strains can be maintained.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION AND CONCLUSION

Over the last few decades, significant production improvements have been achieved through the application of breeding plans and genetic research in terrestrial agriculture and livestock. The application of genetics in fisheries and aquaculture has been largely neglected. Therefore, fish and other aquatic organisms are less domesticated than livestock (Jhingran and Pullin, 1985).

In an Asian country like Bangladesh from where I have come, fisheries is second to agriculture in the overall agro-based economy and contributes about 3.5% GDP and more than 13% to the nations foreign exchange earnings. Fish accounts for about 6% of the per capita protein intake and contributes 80% of the animal protein intake of the people. Unfortunately a consequent decline in fish production from inland waters and an increase in population size has reduced per capita consumption of fish from 33 g/day/person in 1963-64 to about 20.8 g/day/person in 1988-89 a decrease of nearly 12 g/day/person. As a result the amount of animal protein is very low in the every day diet of our people (Rahman, 1986). According to the report of ICLARM (1991), it is not only in Bangladesh but the whole developing world that the problem of protein malnutrition is increasing. Increased production of fish will be one of the major sources of protein to supply the ever increasing world population. Efficient breeding and genetic research programmes can make a major contribution to increasing productivity and profitability from fish farming. Genetic improvement studies have wider implications in aquaculture than in agriculture (Wilkins, 1981).

Genetic improvement of fish is a wide area, the commercially important fish like tilapia in developing countries offer considerable scope for rapid progress in breeding and production through systematic genetic improvement efforts (ICLARM, 1991), genetic manipulation research is one of the vital approach towards this goal. In the present research *O. niloticus* is a model species, the contributions and results made by the genetic manipulation research project of this fish have been discussed in detail in previous chapters of this thesis (see Chapter 3, 4 and 5 particularly). Therefore, this chapter is a unified view of the contributed results of the manipulation and their implications in tilapia aquaculture as well as a light on the need for further genetic research in tilapia.

6.1 IMPLICATIONS OF THE PRESENT RESULTS IN TILAPIA AQUACULTURE

Like salmonids and some other fish, production of expectedly sterile tilapia by genome manipulation techniques has attracted considerable attention in recent years (see Chapter 3). No work has yet been reported on the identification of the most effective optima of various triploid inducing agents in a single species. The present study was carried out to identify treatment optima for triploidy induction in *O. niloticus* by altering intensity, duration and timings of applications of pressure, heat and cold shocks and uses, in this instance, perturbation of meiotic division of eggs as an experimental model. Since heat shocks, by accelerating development, have the effect of narrowing the window of opportunity for triploidization this makes timing of application of shock more critical. Cold shocks apparently result in much greater inter-individual responses. Hydrostatic pressure was found to be the best technique for producing high and consistent triploid yields. As hydrostatic pressure treatments have some advantages over the heat and cold shocks and proved to be a very predictable and reliable method, it is likely to be widely applied in the laboratory

and field for inducing high rates (100%) triploidy in *Oreochromis* spp. for research and their culture. The main disadvantage is that a hydrostatic pressure machine is costly to purchase at present, making it less available for many developing countries.

In tilapia, the gonadal development and sexual maturation of triploids was unclear before this study. Some authors (Penman et al., 1987a) previously suggested that the over all reduction in gonads in triploid *Oreochromis* spp. of either sex may not be as great as that observed in salmonids. In *O. niloticus*, the nature of sexual development and sterility of triploid female and male fish has been shown by the histological and endocrinological studies in this work. Despite less advantage in growth performance, the complete suppression of ovarian development and sexual maturation of females and functional and reproductive sterility of males observed in this fish may be of some benefit in aquaculture. Sterility is a potential factor for fish farming, where production cycles extend into maturity, stocking of these fish into production systems especially in pond culture would eliminate overpopulation and uncontrolled reproduction. Therefore, it is expected that triploid tilapia might show better performance under natural pond conditions as opposed to laboratory based tanks. The reason is that in natural conditions diploid tilapias will spawn frequently and the grow-out ponds will be heavily overcrowded with undesirable young fish, they will compete with each other for available space and foods and will ultimately be stunted. This situation should not happen in the case of ponds stocked with completely sterile triploid populations. The use of sterile triploid tilapia should also be the technique of choice if there is a risk of gene introgression of farmed stocks into native wild stocks. The widespread use of induced triploid is limited at present until methods and commercial facilities to produce large number of triploid tilapia stocks at a reasonable cost can be established.

Induction of gynogenesis by inhibition of the first cleavage at mitotic division of a zygote is considered to be more useful and promising method for producing inbred lines than meiotic gynogenesis as individuals will be homozygous at every gene locus (for reference see- Chapter 4). Methods for the suppression of first cleavage and production of mitotic gynogenetics in *O. niloticus* using pressure and heat shocks have been successfully developed in the present study. Mair (1988) stated that the development of a reliable technique for the production of mitotic gynogenetics where completely homozygous fish can be produced opens up possibilities for genetic improvement of tilapia stocks. Mitotic gynogenetic fish have been used to study induced mutations at specific loci and lethal mutations (Chakrabarti et al., 1983; Walker and Streisinger, 1983; cited by Ihssen et al., 1990). The results of the present study (Chapter 4) suggest that the first generation of mitotic gynogenetics have limitations related to their reduced viability in growth rate, reproductive performances, and increased variability for aquaculture. But they are potentially valuable as broodstock to produce a second generation of clonal lines.

Both outbred and inbred clones of *O. niloticus* were successfully produced for the first time in tilapia using the methods developed in this research. A model for the production of clonal lines has, therefore, been proposed (Chapter 4). Outbred (F_1 hybrids) and inbred (homozygous) clones are expected to be suitable materials for various physiological, immunological and genetic investigations. They may help in detecting recessive mutations or insertion mutants in trangenesis studies and linkage between genetic markers and quantitative trait loci (Stuart et al., 1989; Beckmann and Soller, 1983; cited by Quillet et al., 1991). Clones are valuable products for fixing novel and superior genes in a line which could be utilitized for desirable selective breeding and genetic improvement of tilapia

stocks. It is expected that such vigorous clones will also be of great use as a pure "gene pool" for heritability and sex differentiation studies as well as developing breeding schemes based on the exploitation of heterosis. Commercial applications of these lines in tilapia aquaculture is obvious but the recent nature of laboratory oriented work and lack of follow up trials makes this unlikely in the near future.

The Mendelian mode of body colour inheritance has been extensively studied in mutant *O. niloticus* and Thai red tilapia as one part of the research work for this thesis. In both the strains red body colour was found to be an autosomal dominant trait over wild type. But the existing broodstock population are a mixture of both homozygtes (RR) and heterozygotes (Rr). If both the red mutant strains are desired to be true breeding in a population, they should obviously be fixed as all homozygous at the "R" allele, the undesirable "r" allele must be culled in some way. Therefore, the Mendelian test-cross technique has been proposed in the light of the experimental results of this thesis in order to develop their true breeding broodstock population for 100 % red tilapia seed production by the hatchery operators and fish farmers.

The dominant "R" gene discovered in both the mutant strains has important implications for certain types of genetic research. Particularly this gene will be a useful colour marker for gynogenetic and androgenetic studies.

6.2 SCOPE FOR FURTHER GENETIC RESEARCH IN TILAPIA

The future of tilapia genetic research appears to have great potential. Development of precise research methodologies to produce improved tilapia breeds have just begun. Much greater team work between tilapia breeding biologists and the geneticists are essential in the near future to develop better commercial strains of tilapia suitable for different farming conditions in Asia and Africa. Apart from or in addition to the results of tilapia genetic manipulation research of previous and present work, there are some other interesting areas for further genetic improvement of tilapias as follows:

i) As triploid male *O. niloticus* have been found to be functionally and reproductively sterile (see Chapter 3), such sterility can be of interest in itself, quite apart from other physiological and morphological considerations. Therefore, it is necessary to study the courtship behaviour of triploid males with diploid females in the presence of diploid males. If triploid males can compete successfully with diploid males, then the option will come to introduce them (triploid males) into a wild or control population to suppress the undesirable natural reproduction like other organisms (particularly the Mediterranean fruit fly *Ceratitis capitata*, cited by Ihssen et al., 1990). This technique can be applied in tilapias or other important culturable fish, where control of natural reproduction is some time very essential for the benefit of their aquaculture.

ii)Induction of mitotic gynogenetics and production of their clones in *O. niloticus* have been successful in the present project of this thesis (Chapter 4), therefore, further research could be initiated to develop or apply the technique for other commercial tilapia strains. Research on clonal lines could be expanded involving studies on the growth, other phenotypic traits,

investigation of sexual development and relative endocrine profiles of outbred and inbred clones. Since such clones are genetically uniform a reduction in phenotypic variation and increased or superior developmental stability is expected (Komen et al., 1991b). Interesting research with clones as standardised animals could also be carried out on disease resistance, heritability, immuneresponse and sex differentiation studies. Clones could be used as controls in selection experiments particularly in commercial testing.

iii) An alternative and similar approach to mitotic gynogenesis is the production of androgenetic diploids in tilapia. Again this would of interest in the production of homozygous "inbred lines" or "clones" in two generations. Similar late pressure and heat shock parameters used in the gynogenetic work for the suppression of first cleavage of eggs in *O. niloticus* should induce androgenesis although the UV irradiation of the eggs may result in some change in these parameters or they may vary due to the cytoplasmic constituents contributed by sperms being different. Production of an all male population in tilapia by androgenetic technique would have great advantages for commercial applications to replace hormonal sex-reversal. Another possible direct application of androgenesis may lie in recovering of genotypes from cryopreserved sperm, this is important as eggs and embryo cryopreservation have not yet been successful in fish (Stoss, 1983; cited by Thorgaard, 1986).

iii) There are a few reports on induction of tetraploidy in tilapia (see Review of Literature in section 1.3.1) but none about the viability of tetraploids as in rainbow trout (Chourrout et al., 1986). As induced triploid production of tilapia has some limitation in commercial application, so, further research is needed to develop viable tetraploids for diploid x

tetraploid crosses to produce sterile hybrid triploids. The late pressure and heat shock techniques developed for the inhibition of mitotic cleavage of eggs in the present study may be useful to induce viable tetraploids in tilapia.

iv) It has been seen from the present study that first mitosis in tilapia eggs can easily be blocked using physical shock treatments. Unlike other branches of agricultural science, chromosome engineering has been slowly progressed in fish and other aquatic animals. Now there is a great scope for research to develop valuable insights into the architecture of the spindle and know the structure changes as mitosis progresses in fish eggs. This study will certainly be helpful for further improvement and discovery of new findings in fish genetic manipulation science.

v) DNA fingerprinting techniques have been successfully applied in humans and it is becoming increasingly important for animals (Hill, 1987; cited by Mair, 1988). Application of DNA fingerprinting in fish is in its infancy (Carter et al., 1991). Research in this field using DNA probes will open up a new avenue to analyze and estimate the degree of inbreeding associated with the two types of induced gynogenetics (meiotic and mitotic) as well as of natural populations. According to Mair (1988), DNA probes have now been isolated that hybridize to a single locus and ultimately these locus-specific probes may be of value in establishing linkage to genes affecting important traits such as growth rate and age to maturity of fish like tilapias.

vi) Introduction of novel genes into mouse, amphibians and fruit fly is a recent development. This can also be done in fish. Transgenic individuals have been produced in

rainbow trout (Mclean et al, 1987) and tilapia (Brem et al., 1988; Mr. Azizur Rahman pers. communication), but no report has been found yet on the expression of a foreign gene in fish. Therefore, further research in this area is immediately needed which might benefit in rapid genetic improvement of tilapia strains.

vi) Another important research option lies in genetic selection programmes as recently proposed by ICLARM (1991). Because of generations of inbreeding and introgression of poor genes from undesirable strains, genetic deterioration of the existing cultured stocks of tilapia has made many of them unsuitable for aquaculture. Selective breeding programmes will be one of the most useful method of improving desirable traits in a founder stock with high genetic variability which might have ultimately benefit to increased growth rate and survival of commercially important tilapia strains such as *O. niloticus* and several red tilapias. As there is evidence that the Norwegian salmon industry has increased its productivity by 60 - 70% by applying selection, improved feeding and systematic management programmes (ICLARM, 1991).

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APPENDICES

APPENDIX 1

A. WRIGHT'S BLOOD STAIN

Solid dye	0.3 g
Methanol	100.0 ml
Glycerol	3.0 ml

B. SORENSEN'S BUFFER

Sodium bi-phosphate	9.47 g
Distilled water	1.00-1
pH	7.0

C. 0.01M PHOSPHATE BUFFER (FOR CHROMOSOME PREPARATION)

0.5M KH_2PO_4 stock solution	6.26 ml
0.5M Na_2HPO_4 stock solution	4.56 ml
Add distilled water	500 ml
PH	7.0

D. 0.5M KH_2PO_4 STOCK SOLUTION

6.8 g KH_2PO_4 salts in 100 ml water

E. 0.5M Na_2HPO_4 STOCK SOLUTION

17.9 g Na_2HPO_4 salts in 100 ml water

APPENDIX 2

BUFFERS AND STAINS FOR ELECTROPHORESIS

A. BUFFERS

TEB Buffer

Tris	60.57 g
EDTA	5.99 g
Boric acid	15.00 g

Dissolve in distilled water to make volume 1000 ml, adjust pH 8.5.

For electrode use undiluted, for gel dilute 1:10 with distilled water.

CTC Buffer

Tris	30.29 g
Citric acid	15.76 g

Dissolve in distilled water to make volume 1000 ml, adjust pH 8.0.

For electrode use undiluted, for gel dilute 1:25 with distilled water.

B. STAINS (FOR TILAPIA SAMPLES)

ADA (Adenosine deaminase)

Adenosine	15 mg
MTT	200 µl
PMS	20 µl
XOD	4 µl
NP	10 µl

Mix with 25 ml .05M PO_4 buffer (pH 7.8) and add 20 ml 2% boiled agar (50 - 60 °C).

APPENDIX 2 (CONTD.)

EST (Esterage)

Soak gel slice in either 0.05M PO_4 (pH 6.5) or 0.1M tris maleate (pH 5.3) buffer, leave at 4 °C for 15 minutes, then remove buffer and add stain as follows:

Fast blue	20 mg
α napthyl acetate	10 mg

Dissolve in 1 ml acetone and mix 25 ml buffer as above and add 20 ml 2% boiled agar (50 - 60 °C).

AAT (Aspartate aminotransferage)

Tris	300 mg
L-Aspatic acid	65 mg
α Ketoglutaric acid	20 mg
Pyridoxal-s-phosphate (PSP)	10 mg
Polyvinyl polypyrrolidone (PVP)	10 mg
Fast blue RR salt	25 mg

Dissolve in 25 ml distilled water and add 20 ml 2% boiled agar (50 - 60 °C).

MEP (Malic enzyme)

Malic acid	100 mg
$MgCl_2$	500 µl
NADP	5 mg
MTT	200 µl
PMS	20 µl

Mix with 25 ml 2.4% tris solution (0.6 g tris in 25 ml dissolved water) and add 20 ml 2% boiled agar (50 - 60 °C).

APPENDIX 2 (CONTD.)

AH (Aconitate hydratase)

Cis Aconitic acid	75 mg
$MgCl_2$	3 µl
NADP	5 mg
IDH	200 µl
MTT	200 µl
PMS	20 µl

Mix with 25 ml 0.4M tris HCL (pH 8.0) and add 20 ml 2% boiled agar (50 - 60 °C).

FH (Fumarate hydratase)

Fumaric acid	60 mg
NAD	20 mg
Sodium pyrovate	20 mg
MDH	7.5 µl
MTT	200 µl
PMS	20 µl

Mix with 25 ml 0.5M tris HCL (pH 8.0) and add 20 ml 2% boiled agar (50 - 60 °C).

C. FIXING SOLUTION FOR STARCH GEL STAIN

Acetic acid (glacial)	200 ml
Methanol	1000 ml
Distilled water	1000 ml

Mix thoroughly.

APPENDIX 3

A. HISTOLOGICAL PROCESSING SCHEDULE FOR AUTOMATIC TISSUE PROCESSOR

50% methylated spirit	1 hours
80% methylated spirit	2 hours
100% methylated spirit	2 hours
100% methylated spirit	2 hours
100% methylated spirit	2 hours
Absolute alcohol	2 hours
Chloroform	2 hours
Chloroform	1 hour
Chloroform	1 hour
Paraffin wax	2 hours
Paraffin wax	2 hours

B. HAEMATOXYLIN-EOSIN STAINING PROTOCOL FOR HISTOLOGICAL SLIDES OF TILAPIA GONADS

Xylene	5 minutes
Absolute alcohol	2 minutes
Methylated spirit	1.5 minutes
Water (wash)	0.5 minute
Haematoxylin	5 minutes
Water (wash)	1 minute
1% acid alcohol	4 quick dips
Water (wash)	1 minute
Scott's tap water substitute	0.5 mimute
Water	Wash well
Eosin	0.5 - 1 minute
Water	Wash quickly
Methylated spirit	30 seconds
Absolute alcohol	2 minutes
Absolute alcohol	1.5 minute
Xylene	5 minutes
Mounting	DPX

APPENDIX 4

A.

	CORTLAND SALT SOLUTION (Wolf, 1963) (gl^{-1})	MODIFIED CORTLAND SALT SOLUTION (gl^{-1})
NaCl	7.25	1.88
$CaCl_2.5H_2O$	0.23 (0.17*)	0.23 (0.17*)
KCl	0.38	7.20
$NaH_2PO_4.2H_2O$	0.41 (0.36**)	0.41 (0.36**)
$NaHCO_3$	1.00	1.00
$MgSO_4.7H_2O$	0.23	0.23
Glucose	1.00	1.00

Dissolve in 1000 ml distilled water, PH 6.8 - 6.9

*for dihydrate salt
** for anhydrous salt

B. ACETO-CARMINE STAIN (Guerrero and Shelton, 1974)

Carmine	0.5 g
45% Acetic Acid	100.0 ml

Boil for 2 - 4 minutes, cool and filter.

APPENDIX 5

PAPERS PUBLISHED FROM THIS THESIS

1. Hussain, M.G., Chatterji, A., McAndrew, B.J. and Johnstone, R., 1991a. Triploidy induction in Nile tilapia, *Oreochromis niloticus* L. using pressure, heat and cold shocks. Theor. Appl. Genet., 81:6-12.

2. Hussain, M.G., Penman, D.J., McAndrew, B.J. and Johnstone, R., 1992a. Suppression of first cleavage in the Nile tilapia, *Oreochromis niloticus* L.- a comparison of the relative effectiveness of pressure and heat shocks. Aquaculture (in press).

3. Hussain, M.G., Penman, D.J. and McAndrew, B.J., 1992b. Effects of triploidy on the sexual maturation and reptoduction in Nile tilapia, *Oreochromis niloticus* L. In: The proceedings of the 3rd International Symposium on Tilapia in Aquaculture (ISTA III), 11 - 16 November, 1991, Abidjan, Ivore Coast (in press).

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