Genetic analysis of specific and non-specific immune response in *Oreochomis niloticus* L.

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by

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THIS VOLUME HAS A VERY TIGHT BINDING

Dedicated

to

my parents, my wife Shanjita and son Asif

ABSTRACT

Gynogenesis or androgenesis were used to produce completely homozygous inbred fish, from which clonal lines were established by subsequent gynogenesis, androgenesis and sib mating. Homozygosity of the inbred lines was verified using multilocus DNA fingerprinting and isozyme locus *ADA**, which showed all gynogenetic offspring to have only maternal inheritance. DNA fingerprints of meiotic and mitotic gynogens showed bands consistent with those of their mother but not of their father. Inbred clones also showed identical banding patterns with their mitotic mother and not their father. The outbred clones, in contrast, shared bands with both parents according to Mendelian inheritance.

Sex ratios of both meiotic and mitotic gynogenetics were analysed and a significantly higher number of females ($P<0.05$) were found in both gynogenetic groups. All inbred clones, except one line, were100% females.

MHC class II B genotypes of the clonal lines were determined by PCR. Scale grafting was carried out between clonal lines where two sets of grafts were reciprocally exchanged. Both sets of grafts were completely rejected by reciprocal recipient fish. The mean survival time of the second set of grafts was significantly shorter than that of the first set. This suggested that the differences in the MHC haplotypes of different clonal lines exerted strong alloantigenic effects on the foreign grafts which led to their rejection and memory in immune system.

Non-specific immune response of different inbred and outbred clonal lines was examined by analysing an array of immunological parameters. Significantly different serum lysozyme activity ($P<0.05$) was found between inbred clonal lines. The outbred

clonal lines showed an intermediate level of lysozyme activity to that of their parents. Significant differences were found between the number of macrophages containing 0 and $\geq$ 10 phagocytosed bacteria between the different groups. In the artificial challenge, significantly different susceptibility to *A. hydrophila* infection was found between inbred clonal lines. The outbred clonal lines showed an intermediate infection level between that of their parents, and their disease resistance was higher than that of the least resistant parent. The artificial challenge results revealed that there was a genetic difference in the disease resistance between different clonal groups of fish. It also suggested that disease resistance of an outbred population might be improved by crossbreeding.

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1. General Introduction

1.1 Introduction

Over the last few decades, aquaculture has been practised to make up the shortfall from wild fisheries. According to the intensity of aquaculture, it can be categorised into three groups : extensive, semi-intensive, and intensive aquaculture. In the past it was mostly extensive aquaculture which was practised, but due to the development of culture facilities, for example, hatcheries, different culture units, feeds, water quality management aids, semi-intensive and intensive aquaculture systems are now being practising in most parts of the world. It is well known that in the last few years, world fish production from both wild capture and aquaculture has increased considerably but the quality of fish from wild capture has been declining over this period. There are many factors, which may be responsible for the degradation of fish quality, and continuous exploitation of natural stocks is to be considered the main one. It reduces the vulnerable fish population and consequently low value or poor species are often being captured. In the case of culture fisheries, aquaculture has been expanded mainly due to technical advances but very limited effort to manage the genetic status of fish is being used. As a result, loss of genetic variation is common in poorly managed cultured strains. Genetic variation is considered as the raw material for evolution and selection. Exploitation of genetic variation through selection and modern genetic manipulation techniques has led to huge improvements in other species but in fish this has been limited to a few species, such as GIFT (Genetically Improved Farmed Tilapia) (Eknath *et al.* (1993) and salmon (Kinghorn, 1983; Chevassus, 1980, 1992). To maintain the genetic variation within populations, it is essential to manage the broodstock properly. But unfortunately, inbreeding has occurred in most fish hatcheries and has resulted in the reduction of genetic variation within populations. As a result the

progenies often face growth difficulties, such as a lower growth rate, lack of disease resistance, deformities and early maturation (Kincaid, 1976, 1983; Bondari and Dunham, 1987; Tripathi and Khan, 1990).

In intensive culture, fish are usually stocked at relatively high densities and this is known to cause problems of stress and disease. Disease can affect or sometimes totally destroy whole fish stocks. There are at least four basic measures to reduce or control fish disease: i) treatment with drugs, ii) killing of diseased fishes and replacing stocks after disinfection, iii) vaccination, and iv) improvement of disease resistance by selection or genetic manipulation. The first three measures have been frequently applied (Hayes, 1984; De Kinkelin and Michel, 1984; Lamers, 1985), while little information is available on the fourth. The concept of genetic resistance is receiving a lot of attention at the moment since the other methods described are facing a variety of setbacks. In the case of drug treatment, the antibiotic oxytetracycline, fed orally, has been approved for specific bacterial diseases. However, experimental results show that this drug could be immunosuppressive for thymus-dependent immune responses in carp (Rijkers *et al.*, 1980; Grondel and Boesten, 1982). A similar effect has been found in rainbow trout. For the prevention of fish diseases, vaccination has been suggested as an alternative to treatment with antibiotics. Although some successful results of vaccination of Atlantic salmon against furunculosis have been reported (Erdal and Reitan, 1992), vaccines have not yet been effectively produced against all fish diseases.

Fish possess a variety of defence mechanisms against infection and these include a mechanical barrier (skin), a non-specific immune system such as lysozyme, interferon, complement and phagocytic cells, and a specific immune system. It is known that the specific immune system of mammals, birds and amphibians are controlled by a group of closely linked genes called the major histocompatibility complex (MHC) (Gotze, 1977;

Flaherty and Cohen, 1982). Like mammals, fish also have a distinct MHC-controlled immune system which has been determined from skin transplantation and mixed lymphocyte reaction experiments (Rijkers, 1982; Komen *et al.*, 1990).

The MHC in fish is composed of two major classes of genes, class I and class II, and these genes are located on closely related chromosomal regions. Recent studies on the MHC of fish revealed that class I and class II loci are located in different linkage groups and these two molecules segregate independently (Stet *et al.*, 1998). The MHC genes are polymorphic in nature so that each gene possesses many alleles. These MHC alleles are inherited from each parent to their offspring as a set known as a haplotype. Unlike MHC in higher vertebrates, fish MHC is less studied and its haplotype characteristics are yet to be fully elucidated. A recent study determined the presence of MHC in cichlids and so far 21 different haplotypes have been identified (Edward Malaga, Personal communication). In the present study, different clonal lines of *Oreochromis niloticus* were produced and their MHC haplotypes were identified by PCR amplification and sequencing. Klein (1986) suggested that MHC haplotypes are closely linked to disease resistance. MHC genes have been shown to be responsible for disease resistance in humans (Silver and Goyert, 1985), mice (Quddus *et al.*, 1986) and chickens (Maijala, 1984). Since fish elicit an immune response against foreign antigens, it might be argued that particular MHC haplotypes along with other resistance genes enable to protect them from disease. Like other vertebrates, it can be expected that by studying fish MHC genes, certain MHC haplotypes relating to disease resistance could be identified and those haplotypes could be used to produce more disease resistant strains of fish.

1.2 Importance of selection and genetic manipulation in aquaculture

The application of selection and genetic manipulation techniques in agriculture, both for plants and livestock, has been shown to increase production. In the last few decades, many high yielding and disease resistant strains have been developed for agriculture. In aquaculture, genetic manipulation is comparatively new and less frequently applied. In recent years, a variety of genetic manipulation techniques have been applied to different fish species and in some cases, satisfactory results have been obtained. However, these techniques are still mostly confined to laboratory studies.

Until now, the most studied component of fish genetics is chromosome set manipulation. Biologically fishes are oviparous animals and this characteristic presents opportunities for manipulating their chromosome numbers. By manipulating chromosome sets, it has been possible to produce haploids, polyploids (triploids and tetraploids), and gynogenetic and androgenetic dipoloids in many fish species. The triploid fishes are expected to be functionally sterile and this has been shown in the case of tilapia (Penman *et al.*, 1987b; Hussain, 1992). The production of triploid sterile tilapia has considerable potential in tilapia culture, because it could solve the well-documented problem of early maturation and unwanted breeding (Mair and Little, 1991). As triploids are sterile, they can be cultured in natural waters with reduced adverse environmental impacts (Tave, 1993). However, in tilapia, the technical difficulties associated with large scale triploid production have prevented this from being a commercially feasible technique for aquaculture.

In the last few years, studies on gynogenesis and androgenesis have been attempted with the objective of producing homozygous inbred and clonal lines. The inbred lines can be used for many research purposes such as gene mapping (Allendorf *et al.*, 1986) and standardisation of bioassays in immunological and endocrinological

studies. As well as these areas, gynogenesis and androgenesis can be used in the genetic analysis of traits such as elucidating sex determining mechanisms (Komen and Ritcher, 1993), and to develop techniques for genetic manipulation of the mechanisms to produce monosex populations for culture (Avtalion and Don, 1990, Pandian and Varadaraj, 1990, Mair *et al.*, 1991a, 1991b). Apart from the production of valuable inbred and clonal lines, androgenesis can also be used to recover genotypes from cryopreserved spermatozoa (McAndrew *et al.*, 1993).

Development of disease resistant strain of fish is another promising objective of genetic manipulation techniques. Fish have a distinct immune system which as explained above is controlled by a group of polymorphic genes called the MHC (Rijkers, 1982). As well as MHC genes, fish might possess some other genes which also control the immune response. The immune response genes can be manipulated by producing homozygous inbred and clonal lines through either gynogenesis or androgenesis. Since inbred lines are homozygous at every gene locus for a particular allelic set of genes, they may differ from each other and show variable disease resistance. *In vivo* and *in vitro* analyses of the specific and non-specific immune responses of inbred lines may reveal the nature of different alleles of genes and thus more disease resistant strains can be developed.

In the case of cichlids, the identification of disease resistant strains and using them in selective breeding to produce more resistant individuals is quite new. Selective breeding has been used in salmon, trout and common carp to improve growth (Gjedrem, 1983). Genetic variation in resistance to furunculosis challenge has been reported in Atlantic salmon (*Salmo salar*) and it was suggested that resistance to furunculosis could be effectively improved by selective breeding (Gjedrem *et al.*, 1991). Mass selection for resistance to induced furunculosis in the fingerlings of brook and brown trout greatly

reduced mortalities (Ehlinger, 1977).

Disease resistant traits could also be manipulated from one species to another by gynogenesis using incomplete irradiated sperm. The offspring produced by this way contain some residual paternal inheritance which may show resistance to certain diseases and it has been found in rainbow trout (Thorgaard *et al.*, 1985; Disney *et al.*, 1987).

1.3 Tilapia – a model fish for genetic studies

Tilapia are bony fishes and members of the family Cichlidae. This group is endemic in Africa but many of its members have been distributed in many countries of the world. Many tilapia species are being cultured in different countries but it is generally agreed that *Oreochromis niloticus* is the most promising species for freshwater culture. The major production of tilapia comes from three *Oreochromis* species, *O. aureus*, *O. mossambicus* and *O. niloticus*. Among the three species, *O. niloticus* is the best and preferred both by farmers and consumers, because of its fast growth rate, tasty flesh, deep body and comparatively older spawning age.

For genetic studies, tilapia is considered as a suitable fish species. They have a comparatively short generation time, usually attain sexual maturity at the age of 4-6 months and females spawn regularly at 3-6 week intervals. Sexual differentiation of the gonads (ovary or testis) of a characteristic morphology occurs in *Oreochromis* species around 15 to 30 days after fertilisation at 23 to 25 °C (Nakamura and Takahashi, 1973). Immediately after attaining sexual maturity, and at a suitable water temperature, most tilapia females are able to undergo successive breeding cycles, producing new broods at 4 to 6 week intervals and thus able to spawn throughout the year under the correct

conditions. Tilapia are very easy to spawn and can be reared, grown and matured in aquarium systems (Trewavas, 1983).

O. niloticus can spawn in both natural and artificial conditions. Under natural conditions, the male *Oreochromis* takes sole responsibility for selecting and constructing the nest where spawning takes place (Lowe-McConnel, 1959; Ruwet, 1963). During spawning, the female lays her eggs in the nest and then the male fish swims over the eggs and sheds his sperm on top of eggs. After fertilisation, the female collects all her eggs in the mouth where hatching takes place. The female takes care of her offspring for approximately 20-30 days after spawning and then leaves them.

In laboratory conditions, *O. niloticus* reach to sexual maturity around 6 months and are able to spawn at two week intervals. Artificial spawning can be carried out by stripping eggs from a fully ovulated female and fertilised them with sperm collected from mature males (Valenti, 1975). Generally, tilapia spawn between 20-23 °C and their secondary sexual breeding characteristics become prominent above 20 °C (Fryer and Iles, 1972, cited in Trewavas, 1983). The reproductive cycle of tilapia is usually halted at water temperatures below 20 °C (Huet, 1972, cited in Trewavas, 1983).

In the present study, *O. niloticus* was used as a model species. Among the cichlids, *O. niloticus* is the most widely studied species in the Institute of Aquaculture. Over the last 15 years, many ploidy manipulation techniques such as diploid gynogenesis, androgenesis, triploidy, genetic markers, karyology and experimental approaches of *O. niloticus* have been developed in this Institute (e.g. .McAndrew and Majumdar, 1983; Hussain, 1992; Myers *et al.*, 1995).

1.4 Gynogenesis

Gynogenesis is an artificial reproductive process where by embryonic inheritance is entirely maternal and no genetic material is incorporated into the developing embryo from the paternal side. It is, therefore, necessary to destroy the sperm DNA before they are used to fertilise normal eggs. Artificial gynogenesis was first performed in the frog, *Rana fusca* by Hertwig (1911, cited by Purdom, 1983 and Ihssen, *et al.*, 1990). He observed that when eggs were fertilised with higher doses of gamma irradiated sperm, the development of the embryos was more normal than those fertilised with a lower dose of irradiated sperm. This paradoxical phenomenon known as the "Hertwig effect", enabled optimisation of the irradiation dose so as to produce a high fertilisation rate and large numbers of haploid embryos (Don and Avtalion, 1988a; Penman, 1989).

Different methods have been used for the inactivation of sperm DNA. Earlier works used gamma or X-rays for the inactivation of sperm because they have a high penetration and can be used to treat large quantities of sperm. However, some residual parental characteristics or chromosome fragments have been detected in gamma or X-rays irradiated sperm, which make this type of irradiation unsuitable for gynogenesis work (Ijiri, 1980; Onozato, 1984; Chourrout and Quillet, 1982; Thorgaard *et al.*, 1985; Allen, 1987). Chemical mutagens such as diemethylsulphate or toluidine blue have also been used to inactivate sperm of amphibians and fish (Briggs, 1952; Tsoy, 1969; Chourrout, 1986). As with gamma or X-rays irradiation, supernumerary chromosome fragments were found in the chemical mutagen treated sperm of rainbow trout, *Oncorhynchus mykiss* (Chourrout, 1986). Alternatively, UV light is used to inactivate sperm and has been found to be more effective than other treatments. Chourrout and Itskovich (1983), Mair *et al.* (1987) and Hussain *et al.* (1993) performed gynogenesis with tilapia, *O. niloticus* by inactivating sperms using UV light and found no residual

chromosomal fragments in the resultant offspring. UV light is safer than other ionising rays due to low penetration, but for effective treatment UV requires a thin and transparent sperm medium (Thorgaard, 1983). UV light is not effective on opaque and thick sperm samples and is therefore not suitable for treatment of large volumes of milt. The advantages of using UV treatment to inactivate sperm outweigh the disadvantages in that it is very effective, readily available, easy to transport, has low costs, and is easy and safe to use (Allen, 1987; Chourrout, 1987).

According to Bohm (1891, cited by Ihssen *et al.*, 1990), fish eggs complete their first meiotic division before fertilisation, so only the remaining second meiotic and first mitotic cell divisions can be manipulated. Generally, two methods are used to make gynogenetic haploid eggs into diploids: meiotic gynogenesis and mitotic gynogenesis. Meiotic gynogenesis can be carried out by applying early heat, cold or pressure shocks at the metaphase of the second meiotic division before the loss of second polar body which induces retention of the second polar body. The early shock treatments disrupt the microtubili that make up the spindle during meiosis (Dustin, 1984) and inhibit cellular division (Reinschmidt *et al.*, 1979; Streisinger *et al.*, 1981; Onozato, 1984). Applying short heat shocks at a sublethal level (35-42 °C for 1.5 - 5 mins) has been effective in preventing extrusion of the second polar body and inducing diploidization in a number of warm water fish species, such as zebrafish, *Brachydanio rerio* (Streisinger *et al.*, 1981), common carp, *Cyprinus carpio* (Hollebecq *et al.*, 1986; Sumantadinata *et. al.*, 1990), grass carp, *Ctenopharyngodon idella* (Cassani and Caton, 1985), Indian major carps, *Catla catla, Labeo rohita*, and *Cirrhinus mrigala* (John *et al.*, 1984, 1988), red sea bream, *Pagrus major* (Sugama *et al.*, 1990), and tilapias (Chourrout and Itskovich, 1983; Penman *et al.*, 1987b; Don and Avtalion, 1988b; Mair *et al.*, 1987; Varadaraj, 1990 and Hussain *et al.*, 1993).

Mitotic gynogenesis can be carried out by inhibiting the first mitotic division applying late heat, cold or pressure shocks. Experimental induction of mitotic gynogenesis by interfering with the first cleavage has been conducted with amphibian species. Reindschmidt *et al.* (1979) first produced homozygous diploid *Xenopus laevis* by suppression of the first mitotic division using pressure shock. Streisinger *et al.* (1981) claimed to be the first to produce 100% viable mitotic gynogenetics from zebrafish, where the eggs were fertilised with UV irradiated sperms and treated with heat shock at 22.5-28 mins post fertilisation to inhibit the first mitotic division. Since this time many workers have attempted to produce homozygous diploids in a number of fish species using different shock treatments. Some have successfully produced mitotic diploids in common carp, *C. carpio* (Nagy, 1987; Komen *et al.*, 1991), ayu, *Plecoglossus altivelis* (Taniguchi *et al.*, 1988), medaka, *Oryzias latipes* (Ijiri, 1987), Nile tilapia, *O. niloticus* (Mair *et al.*, 1987; Hussain *et al.*, 1993; Myers *et al.*, 1995), rainbow trout, *Onchorynchus mykiss* (Quillet *et al.*, 1991), channel catfish, *Ictalurus punctatus* (Goudie *et al.*, 1991) and Asian carp, *L. rohita* (Hussain *et al.*, 1994b).

Gynogenesis can be used in the production of inbred lines of fish. As already indicated inbred lines can be useful in many areas of fish culture research, such as gene mapping (Allendorf *et al.*, 1986), sex determination (Komen and Richter, 1993), and immunological (Kaastrup *et al.*, 1989) and endocrinological studies. Golovinskaya (1968) and Purdom (1969) thought that meiotic gynogenetic diploids could be used for the production of homozygous inbred lines, but the retention of the second polar body during meiotic gynogenesis made it uncertain. Because the suppression of the second polar body extrusion during meiotic division results in heterozygosity at many loci due to the occurrence of recombination between chromatids at the first meiotic division and the retention of both non-sister chromatids at the second meiotic division (Purdom,

1969; Nace *et al.*, 1970; Hussain *et al.*, 1994a). Mitotic gynogenesis can be used to produce completely homozygous inbred lines in the first generation and clones in the second. In this process, mitotic gynogenetics are produced by blocking the first mitotic cleavage of eggs which dramatically shortens the generation time to induce inbreeding and produce fully homozygous individuals. In contrast, completely homozygous inbred lines can not be obtained even after repeated meiotic gynogenetic reproduction or conventional methods of sib mating for 10-20 generations (Nagy and Csanyi, 1982; Han *et al.*, 1991; Purdom and Lincoln, 1973). As the inbred lines are free from the recessive lethal and deleterious alleles, they can be used for selective breeding and improvement of fish stocks (Han *et al.*, 1991).

Gynogenesis can also be used to determine sex mechanism in fish. In case of homogametic female like *O. niloticus* (XX), *O. mossambicus* (XX) and silver barb, *Puntius gonionotus* (XX), all-female progeny can be produced by applying gynogenesis (Penman *et al.*, 1987a; Mair *et al.*, 1991a; Pongthana *et al.*, 1995). During production of mitotic gynogens in *O. niloticus*, some mitotic males (XX) might be produced (Hussain *et al.*, 1993; Mair *et al.*, 1991a). These males can be used to produce all-female population by crossing them with any ordinary females. Gynogenetic sex reversed males (XX) can be produced by administering masculinised hormone treatment and these males can be commercially used to produce all outbred monosex female population by crossbreeding where the growth rate of females are superior to males, particularly in salmonids and cyprinids. Sex reversed males can also be used to analyse genetic traits such as identification of sex determining mechanism in a species.

1.5 Androgenesis

Artificial androgenesis is a uniparental reproductive system which is the opposite of gynogenesis and involves fertilisation of genetically inert eggs with normal sperms. As a result, the embryonic development proceeds with the incorporation of only paternal genetic materials. The inactivation of the egg genome can be accomplished by gamma, X-rays or UV radiation. Due to high penetration gamma and X-rays are widely used for egg irradiation (Purdom, 1969; Parsons and Thorgaard, 1985). Myers *et al.* (1995) successfully produced haploid androgens in Nile tilapia, *O. niloticus* by irradiation of eggs using UV light.

Since haploids are not viable they need to be made diploid and androgenetic diploidization can be induced by suppression of the first mitotic division of the egg. Gillespie and Armstrong (1980, 1981) first produced androgenetic diploids in the Mexican axolotl, *Ambystoma mexicanum* by inhibition of first mitotic division using heat shock (36-37 °C for 10 mins) or pressure shock (14, 000 p.s.i. for 8 mins) shortly after fertilisation of eggs. The induction of androgenetic diploids in salmonids were reported by Parsons and Thorgaard (1985); Scheerer *et al.* (1986) and May *et al.* (1988). Grunina *et al.* (1991) successfully induced androgenetic diploids in common carp using late heat shock (40-41 °C) and also produced homozygous androgenetic clones by subsequent androgenesis utilising sperm from the androgenetic males.

Spontaneous androgenetic diploids among interspecific hybrids was reported by Stanley (1976, cited by Ihssen *et al.*, 1990). In this study, androgenetic diploid grass carp, *C. idella* were obtained from crosses between common carp female and grass carp male and the diploids might be generated from genetic incompatibility of two genomes resulted in removal of the female's pronucleus. Spontaneous androgenetic diploids were also observed in common carp, *C. carpio* (Gomelsky and Recoubrastsky, 1991).

Although artificial androgenesis is successful under laboratory conditions, the poor survival of diploid androgens is still a big constraint. According to Scheerer *et al.* (1986) there are a number of factors that might be involved in the poor survival of diploid androgens, such as the genotype of the sperm, damage of the eggs during irradiation and shock treatment. However, androgenesis is a very powerful technique to produce homozygous inbred lines in the first generation and clones in the second. In the case of the Nile tilapia, *O. niloticus*, the genotype of inbred male androgens is YY and they can be used to produce all male progeny in the subsequent crosses with any ordinary females. These fish can be used in subsequent experiments to produce large numbers of YY males for commercial purposes.

Both artificial gynogenesis and androgenesis can be used to produce clonal lines. Gynogenetically, clones can be produced from mitotic females by using meiotic gynogenesis. Clones can be produced from androgenetic males by another round of androgenesis or gynogenesis if they are females. As clones produced by both ways are homozygous for every gene locus, each clonal line possesses a unique set of genes and results in individual line-specific characteristics. For this reason, clonal lines are considered as valuable research tools and can be used for different research purposes such as identification of sex determination mechanism, standardisation of bioassays of immunological and endocrinological studies. It has been observed that clonal individuals are free from recessive deleterious and lethal alleles (Han *et al.*, 1991). Clones produced in common carp, *C. carpio* showed a reduced variation for a variety of morphological traits and superior viability as a result of decreased lethal genes (Komen *et al.*, 1991).

Clonal lines can be used to produce monosex populations by crossbreeding. In *O. niloticus*, sex reversal of gynogenetic clones with 17 α-methyltestosterone hormone

treatment produces neomales (XX) (genotypically female but phenotypically male) and these neomales can be used to produce all-female populations by crossing with ordinary females. The neomales can also be used to produce outbred clonal lines that can be used to analyse genetic traits such as identification of sex determining mechanism and effects of heterosis. Likewise, androgenetic males (YY) or sex reversed androgenetic females, neofemales (YY) can be used to produce all-male fish in *O. niloticus*. In this study both gynogenesis and androgenesis were used to produce clonal lines. In immunological studies, both inbred and outbred clonal lines are expected to show variable immune responses in both *in vitro* and *in vivo* analysis and thus disease resistant clonal strains can be identified.

1.6 Major histocompatibility complex genes

1.6.1 The Major histocompatibility complex genes in mammals

The major histocompatibility complex (MHC) contains a tightly linked cluster of genes whose products are associated with intercellular recognition of self or non-self proteins. The MHC complex is a region of multiple loci that play a major role in the initiation of immune response against invading pathogens. It plays a central role in the development of both humoral and cell-mediated immune responses. The MHC genes encode membrane proteins which are essential to the function of the immune system of all vertebrates (Klein, 1986). The MHC proteins are intimately involved in antigen presentation of the immune system. T lymphocytes can only recognise antigen when it is associated with a MHC molecule. It is evident that MHC molecules both participate in and control physiological immune responses, such as those involved in the recognition and elimination of virus infected cells.

MHC genes code three families of glycoproteins are known as class I, class II and class III molecules (Klein, 1986). The class I and class II molecules are sometimes referred to as MHC antigens or alloantigens, because they can be recognised by the immune system. The class I and class II molecules are mainly expressed as membrane glycoproteins, whereas the products of the class III genes are usually soluble proteins. Class III molecules are associated with the immune process including soluble serum proteins, components of the complement system, and tumor necrosis factors.

The loci constituting the MHC are highly polymorphic. The MHC loci are also closely linked and there is little recombination between these loci. For this reason, an individual inherits the MHC alleles as two sets, one from each parent and each set of the alleles is referred to as a haplotype. In an outbred population, the offspring are generally heterozygous for many loci and inherit both maternal and paternal MHC alleles. As a result, the alleles are co-dominantly expressed in each cell of the offspring. In the case of an inbred population however, each MHC locus is homozygous and all inbred offspring express identical haplotypes.

It has been known that there is a relationship between MHC haplotypes and susceptibility to infectious disease. Among the diseases associated with particular MHC alleles are a large number of autoimmune diseases, certain viral diseases, disorders of complement system, certain neurological disorders and some types of allergies. However, in most MHC-associated diseases, a number of other genes and external environmental factors may also play a role, therefore, it is difficult to determine the actual relationship between specific diseases and MHC haplotypes.

A number of hypotheses have been adopted to determine the role of the MHC alleles in infectious diseases. Susceptibility of an animal to a given pathogen may reflect the role of particular MHC alleles in responsiveness or non-responsiveness to the

pathogen. Variations in antigen presentation by different MHC alleles may determine the effectiveness of the immune system to a given pathogen. If major epitopes (binding site of antigen) on a given pathogen are similar to certain self-MHC molecules, an animal may lack functional T cells specific for those epitopes. Various MHC alleles may also code for binding sites for specific viruses, bacteria, or other products.

Some evidence suggest that a reduction of MHC polymorphism within a species may increase the possibility of its susceptibility to disease. The increased susceptibility to various diseases of a species may result from a reduction in the number of different MHC molecules available to the species and a corresponding limitation on the range of processed antigens with which these MHC molecules can interact. Therefore, the high level of MHC polymorphism that has been found in various species may be advantageous by providing a broad range of antigen-presenting MHC molecules. MHC polymorphism ensures that at least some members of a species will be able to respond to any of a very large number of potential pathogens. Thus MHC diversity appears to protect a species from a wide range of infectious diseases (Kuby, 1997).

1.6.2 The major histocompatibility complex in fish

The major histocompatibility complex encodes cell membrane glycoproteins which appear to be intimately involved in a variety of immunological processes including the restriction of antigen recognition by lymphocytes, the acquisition of the T cell repertoire, and the co-operative interaction among subsets of mononuclear leucocytes. The presence of MHC genes was first discovered by graft rejection where recipients showed their ability to identify non-self grafts. It has been established that all vertebrates including fish show graft rejection and mixed lymphocyte reactions (MLR) (Hildemann, 1970; Kallman, 1970; Nakanishi, 1987a and Nakanishi, 1987b).

The identification of MHC genes in fish has involved a number of conventional approaches including the cross-hybridisation of fish DNA or RNA with probes of higher vertebrates, and the cross reaction of fish cell surface molecules with antibodies against mammalian and avian MHC molecules (reviewed by Kaufman *et al.*, 1990; Stet and Egberts, 1991), but none of them have succeeded. Hashimoto *et al.* (1990) developed generic primers which could be used in PCR for the identification of MHC genes and successfully demonstrated for the first time the presence of both MHC class I and class II genes in carp, *C. carpio*. After this success, MHC genes in a number of fish have been isolated and sequenced. To date, class I A, β_2m, class II A and class II B MHC genes have been isolated from both teleosts and elasmobranches (see Table 1.1).

The analysis of sequences of all MHC genes studied in fish shows that most of the introns are less than 1 kb long. Therefore, short introns seem to be characteristic of fish MHC genes (Ono *et al.*, 1992, 1993c; Klein *et al.*, 1993; Sültmann *et al.*, 1993). The sequences of the class I α_3 domain are comparatively well conserved, whereas the sequences of the class I α_1 and class I α_2 domains have diverged from one another in cyprinid fishes (Okamura *et al.*, 1993). Grimholt *et al.* (1993) and Okamura *et al.* (1993) reported that several amino acids in the peptide binding regions (PBR) are highly conserved. There are also reports that several amino acids which are important for the association with CD4 are conserved within the class II β_2 in carp (Hashimoto *et al.*, 1990), and those for CD8 are conserved within the class I α_3 in banded dogfish, *Triakis scyllia* (Hashimoto *et al.*, 1992). Thus, it is possible that well-conserved amino acids are those important either for peptide binding or in association with CD_4 and CD_8 molecules. These findings along with the data on the polymorphism at the putative PBR, suggest that fish MHC molecules function in a similar to those in mammals.

Table 1.1 Current status of the isolation of MHC genes in fishes (cited from Manning and Nakanishi, 1996)

Species	Genes[a]	Clones	Sequence types	Reference[b]
Teleosts				
Carp	Class I*A*	*Cyca*-ZA	genomic	1
		Cyca-ZA, ZB, ZC	cDNA	2
		Cyca-UA, -TC	cDNA	3
		Cyca-Z	genomic	4
	Class II*B*	*Cyca*-TLAII(YB)	genomic	1
		Cyca-DAB	cDNA, genomic	5
		Cyca-YB	genomic	4
	B2m	*Cyca*-B2m	cDNA	6
Ginbuna	Class I*A*	*Caau*-ZA, -ZD	cDNA, genomic	2
Zebrafish	Class I*A*	*Brre*-UA (~C)A	cDNA, genomic	7
	Class II*A*	*Brre*-DXA	genomic	8
	Class II*B*	*Brre*-DAB1 ~ 4	cDNA	9
		Brre-DA(~F)B	genomic	10
	B2m	*Brre*-B2M, -B2m-G	cDNA, genomic	11
Tilapia	*B2m*	*Orni*-B2m	genomic	6
Cichlid	Class II*B*	*Auha*-M, *Cyfr*-T	cDNA, genomic	12
		Necy-T, *Nili*-M, *Nive*-M, *Pepu*-N, *Pslo*-M, *Psze*, *Thsp*-V	genomic	13
		Meau-, *Mech*-M	genomic	14
Atlantic salmon	Class I*A*	*Sasa*-p23, -p30	cDNA	15
		Sasa-A1-4, -B1	genomic	16
	Class II*B*	*Sasa*-DC, -DB	cDNA	17
		Sasa-DB	genomic	16
Rainbow trout	Class II*B*	*Onmy*-55	cDNA	18
	B2m	*Onmy*		19
Striped bass	Class II*A*	*Mosa*-A	cDNA	20
	Class II*B*	*Mosa*-C, -R,-S	cDNA	21
Perch-like fish	Class II*B*	*Pefl*, *Gyce*	genomic	22
Crossopterygian				
Coelacanth	Class I*A*	*Lach*-UA, UB, UC, UD	cDNA, genomic	23
Elasmobranchs				
Bended dogfish	Class I*A*	*Trsc*-DS	genomic	24
Nurse shark	Class II*A*	*Gici*-DAA, -DBA	genomic	25
	Class II*B*	*Gici*-8, -11	cDNA	27
Atlantic cod	Class I α	*Gamr*- UAA	cDNA	28
	B2m	*Gamr- B2m*	cDNA, genomic	28

[a]The gene designation was shown according to the proposal by Klein *et al.* (1990).

[b]1. Hashimoto *et al.* (1990). 2. Okamura *et al.* (1993). 3. Van Erp *et al.* (1994). 4. Stet *et al.* (1993). 5. Ono *et al.* (1993a). 6. Dixon *et al.* (1993). 7. Takeuchi *et al.* (1995). 8. Sultmann *et al.* (1993). 9. Ono *et al.* (1992). 10. Sultmann *et al.* (1994). 11. Ono *et al.* (1993b). 12. Ono *et al.* (1993c). 13. Klein *et al.* (1993). 14. Ono *et al.* (1993d). 15. Grimholt *et al.* (1993). 16. Grimholt *et al.* (1994). 17. Hordvik *et al.* (1993). 18. Juul-Madsen *et al.* (1992). 19. Shum *et al.* (1994). 20. Hardee *et al.* (1995). 21. Walker and McConnell (1994). 22. Figueroa *et al.* (1995). 23. Betz *et al.* (1994). 24. Hashimoto *et al.* (1992). 25. Kasahara *et al.* (1992). 26. Kasahara *et al.* (1993). 27. Bartl and Weissman (1994). 28. Persson *et al.* (1998).

The MHC molecules are reported to be polymorphic in nature. The MHC alleles differ in their DNA sequences from one individual to another, and consequently have unique structural differences. Polymorphism of MHC genes has been detected in zebrafish, *B. rerio* (Ono *et al.*, 1992), cichlid fishes (Klein *et al.*, 1993; Ono *et al.*, 1993d), Atlantic salmon (Hordvik *et al.*, 1993) and striped bass, *Morone saxatilis* (Walker and McConnell, 1994) at variable regions of the β_1 domain of class II *B* genes and the α_1 domain of zebrafish class II *A* genes (Sültmann *et al.*, 1993) which are associated with residues of the putative PBR. At least five or six alleles with numerous amino acid substitutions at the PBR of class II *A* genes (Kasahara *et al.*, 1993) and two alleles at class II *B* genes (Bartl and Weissman, 1994) have been reported from the nurse shark. These MHC class II alleles function as the mammalian functional class II MHC genes and thus suggest that shark's MHC genes have a functional homology to that of the mammals.

The polymorphic characteristics of MHC genes can be used in population studies. Migration of populations can be determined by using allele frequencies (Klein, 1986). Bugawan *et al.* (1988) reported that the polymorphism of MHC genes can be used to generate DNA fingerprints for individual organisms. Because the pattern of restriction fragments determined on a southern blot using one of these genes as a probe will be virtually unique. DNA fingerprints have already been generated using MHC genes in humans (Martell *et al.*, 1988) and mice (McConnell *et al.*, 1988). Polymorphism of MHC can also be used to improve fish stocks. Klein (1986) reported that MHC haplotypes have a close link to disease resistance. The MHC and disease association in farm animals has also reported by Van der Zijpp and Egbert (1989). MHC haplotypes in human have links to specific autoimmune diseases (Todd, 1990).

1.7 Multilocus DNA fingerprinting

The term DNA fingerprinting was first introduced by Jeffreys *et al.* (1985) to describe a method for the simultaneous detection of many highly variable DNA loci by hybridisation of specific multilocus "probes" to electrophoretically separated restriction fragments. The product of hybridisation of the multilocus probes to the restriction fragments results in a pattern of bands, resembling a bar-code, on an autoradiogram. This pattern is often specific to the individual except in extreme cases of inbreeding or clonal individuals. The bands comprising the fingerprint pattern are inherited in a Mendelian manner (on average half of the bands are derived from each parent), and usually show high somatic and germ line stability.

Multilocus DNA fingerprinting can be produced by two ways: classical hybridisation-based fingerprinting and PCR-based fingerprinting. The hybridisation-based fingerprinting is derived from the exploitation of restriction fragment length polymorphism (RFLP). To produce a DNA fingerprint, complementary probes are used to create complex banding patterns by recognising multiple DNA loci simultaneously. Each of these identified loci is characterised by more or less regular arrays of tandemly repeated DNA motifs that occur in different numbers at different loci. The PCR-based fingerprinting, on the other hand, is produced by *in vitro* amplification of particular DNA sequences with specifically or arbitrarily chosen oligonucleotides ("primers") and a thermostable DNA polymerase. The amplified DNA sequences are separated by electrophoresis and the polymorphic banding patterns are detected by staining.

Multilocus DNA fingerprinting is a very useful technique for genetic studies. It can be used in intrapopulation studies, such as to identify clonal individuals or closely related individuals, and to determine maternal or paternal relationship with their offspring, specially for gynogenetic and androgenetic progenies. DNA fingerprinting can

also be used to estimate inbreeding rates in commercial brood stocks where record keeping is poor or non-existent (Doyle and Talbot, 1986, Eknath and Doyle, 1990).

1.8 Polymerase chain reaction and its application

The polymerase chain reaction (PCR) is a versatile technique which was invented in the mid 1980s (Saiki *et al.*, 1985). After the introduction of thermostable DNA polymerase in 1988 (Saiki *et al.*, 1988), the use of PCR in research has increased tremendously. It is an *in vitro* selective amplification method for specific target DNA sequences from large, heterogeneous genomic DNA. Using a very low amount of DNA (usually a few nanograms), millions of copies of one or more particular target DNA fragments are produced which can be characterised after being electrophoresed and visualised by staining or autoradiography.

At the start of the PCR process, all reaction partners must be single stranded which can be achieved by heat-denaturation. The PCR reaction mixture contains buffer, free nucleotides (dNTP's), and short oligonucleotides as primers, which are usually 15-35 bp long and homologous to the flanking regions of the sequence to be amplified. The reaction mixture also contains DNA polymerase which synthesises a complementary strand by connecting the free nucleotides on the basis of sequence information given by the template strand. Subsequently, the newly formed double-stranded DNA molecules are denatured again and serve as new templates for the next round of DNA synthesis. To complete each PCR cycle needs only a few (normally 2-5) minutes. After approximately 30-40 cycles, the target DNA sequence is theoretically amplified a billion times.

PCR is comparatively easy to use for synthesising DNA molecules measuring up to a few hundred base pairs, the maximum is approximately 10 kb. It is not suitable for amplifying larger DNA molecules. In fish genetic studies, PCR can be used to amplify

specific DNA sequences such as MHC loci, mitochondrial DNA and introduced foreign genes. It can also be used to identify the allelic distribution of a particular gene. The most important advantage of the PCR process is that it needs only a very small amount of DNA and may work even if the DNA is badly degraded. It also works with DNA extracted from dried, buried or mummified samples, and samples embedded or stored in paraffin blocks. Since PCR is a quick method to amplify specific DNA sequences, it was used in this study to amplify MHC class II B genes using two generic primers.

1.9 Graft rejection

The presence of the MHC in mammals was initially identified by graft rejection experiments. Generally graft rejection takes place between two animals that are genetically different and their tissues display significant antigenic differences in MHC loci. MHC loci in mouse H-2, rat H-1 and human HL-A control the production of strong transplant antigens that elicit intense allograft reactions (Amos *et al.*, 1963; Elkins and Palm, 1966). In addition to MHC loci, there are also some other minor histocompatibility loci located on other chromosomes which produce weak transplantation antigens that elicit a relatively mild immune response during allograft rejection (Graff and Bailey, 1973; Hildemann, 1971).

The action of graft rejection is generated principally by a cell-mediated immune response to alloantigens expressed on cells of the graft. It occurs in two phases :

i) a sensitisation phase in which antigen-reactive lymphocytes of the recipient proliferate in response to alloantigens on the graft.

ii) an effector phase in which immune destruction of the graft occurs.

In the sensitisation phase, $CD4^+$ and $CD8^+$ T cells recognise alloantigens on the foreign graft and proliferate in number. They can recognise both major and minor

histocompatibility antigens and respond to both antigens, but their response to the latter is comparatively weak. The response of T cells to major histocompatibility antigens induces recognition of both the MHC molecules and an associated peptide ligand in the cleft of the MHC molecules. To activate the host T_H cells, they require an interaction with an antigen-presenting cell (APC), expressing an appropriate antigenic ligand-MHC molecule complex. There are number of cells which can function as APCs. Usually dendritic cells are found in most tissues, and they serve as the major APC in grafts. APCs of the host can also migrate into a graft and endocytose the foreign alloantigens and present them as processed peptides together with self-MHC molecules.

In the effector stage, a variety of reactions participate in allograft rejection and among them cell-mediated reactions involving delayed-typed hypersensetivity (DTH) and cytotoxic lymphocyte (CTL)-mediated cytotoxicity are the more important. During graft rejection the host's T cells and macrophages influx into the graft in large numbers. Their infiltration is also increased by the influence of cytokines produced by T_{DTH} cells. The recognition of foreign MHC class I alloantigens by host $CD8^+$ cells can lead to CTL-mediated killing. In some cases $CD4^+$ T cells recognise class II alloantigens on the graft and mediate its rejection.

In allografting, the cells present in the grafts sometimes show adverse effect to the recipient which is known as graft-versus-host reaction. This graft-versus-host reaction has been seen in mammals and birds (Clark, 1991) and in amphibians (Clark and Newth, 1972). Nakanishi (1994) recently reported this graft-versus-host reaction in fish. The graft-versus-host reaction may happen when the grafted donor tissue contains immunologically competent cells that the recipient fails to recognise and destroy. The host's inability to recognise the transplanted cells might be due to its immaturity or immunosuppresion, or its genetic constitution. As a result, the grafted cells react against

the host and may lead to excessive damage of other organs and even death. In the case of mammals, donor T cells have an important role in graft-versus-host reactions through the secretion of cytokines by $CD4^{+ve}$ T cells. $CD8^{+ve}$ donor T cells, on the other hand, have a cytotoxic effect on the hosts cells.

Scale grafting in fish is relatively an easy way to test specific immune response. It can be used to determine the expression of different MHC haplotypes. In this study scale grafting has been used to determine the differences of MHC class II B haplotypes between clonal lines and their response to foreign grafts.

1.10 Non-specific and specific immune response in fish

Like mammals, the immune system of fish is divided into the non-specific and the specific immune systems. The non-specific immune system refers to a basic resistance to disease, which the animal is born with i.e. innate immunity. It acts as the first line of defence against infectious agents and is often able to prevent infection by potential pathogens. This type of immunity does not improve with repeated infection. On the other hand, the specific immune response requires the activity of a fundamental immune system, involving cells called lymphocytes and their products. It is based on specific defence mechanisms which improve the host's immunity to a pathogen with repeated exposure, resulting in a specific immunological memory. In general, during infection most of the invading microorganisms are first encountered by a healthy individual's non-specific defence mechanisms without activating the specific response. But when the microorganisms escape from the non-specific host defence, the specific immune response is then activated. Specific immunity does not operate independently of non-specific immunity, rather both defence mechanisms work synchronously, providing a more effective total response.

The non-specific immune system is composed of humoral and cellular defences. The soluble humoral defence factors, such as lysozymes, complement, interferon, cytokines, C-reactive protein, transferrin and lectin, combine with cellular defences such as macrophages, granulocytes, neutrophils, and natural killer cells to provide the animals with innate protection. Lysozyme is widely distributed in vertebrates (Osserman *et al.*, 1974). Many studies have been conducted to determine the role of lysozyme in fish and it has been found to play an important role in the host defence mechanisms against infectious diseases such as Hitra-disease or cold water vibriosis in salmon (Murray and Fletcher, 1976; Lundblad *et al.*, 1979; Lindsay, 1986 and Lie *et al.*, 1989a). It is a hydrolytic enzyme which is able to cleave the peptidoglycan layer of bacterial cell walls and thus destroy the bacteria. Interferon consists of a group of proteins produced by virus-infected cells. It has an ability to bind to nearby cells and induces a generalised antiviral action. Another important component of the humoral defence is complement which comprises of a group of serum proteins that circulate in an inactivated proenzyme state. These proteins can be activated by a variety of specific and non-specific immunologic mechanisms that convert the inactive proenzymes into active enzymes. Once activated, the complement components participate in a controlled enzymatic cascade that results in either destroying the pathogens or facilitating their elimination. Fish complement displays bactericidal activity against non-virulent strains of Gram-negative bacteria, but not against Gram-positive bacteria or virulent strains of Gram-negative bacteria (Ourth and Wilson, 1982; Iida and Wakabayashi, 1983, 1993).

Macrophages of fish are derived from monocytes and are found mainly in tissues and rarely in the blood. They migrate toward the sites of chronic and acute infection where they act as phagocytic cells (Rowley *et al.*, 1988). Non-specific phagocytosis by macrophages has been reported both *in vivo* (Ellis, 1976; Hunt and Rowley, 1986) and

in vitro (Braun-Nesje *et al.*, 1981; Secombes, 1986). Sakai (1984) and Johnson and Smith (1984) determined that opsonization of particles by antibodies and complement fragments facilitated their recognition and engulfment by macrophages.

It is thought that macrophages in the lymphoid tissues function to trap, process and present antigens to lymphocytes. Small metabolically active lymphocytes are associated with the ellipsoid and melano-macrophage (pigmented macrophage) centres of the spleen, and melano-macrophage centres of the kidney (Ellis, 1980).

Granulocytes of fish are another important type of phagocytic cells and are composed of subpopulations of neutrophils, eosinophils, basophils and mast cells. They are generally responsible for the inflammatory response in which they migrate to sites of infection and non-specifically destroy invading microorganisms by phagocytosis or cytotoxic killing (Finn and Nielson, 1971; MacArthur *et al.*, 1984). Soluble proteins such as leukotrienes and complement fragments act as chemoattractants, inducing the granulocytes to the sites of inflammation (Rowley *et al.*, 1988). Normally the number of neutrophils in fish is less than in mammals, but their number increases during stress conditions (Dunn *et al.*, 1989).

The specific immune system of fish comprises cell-mediated and humoral (antibody production) immune responses. In the early 1950s, it was shown that the cells responsible for specific cell-mediated immunity are lymphocytes (Mitchinson, 1953). Initially most of the cell-mediated immunity in mammals was determined by transplantation experiments, particularly the rejection of allogenic grafts. Allogenic graft rejection reveals antigen specificity and immunological memory. These features have provided immunologists with a basic knowledge of the vertebrate specific immune system.

The cell-mediated immune response involves two major groups of cells : lymphocytes and antigen presenting cells. Comment on the fact that macrophages are APC, have non-specific activity but play an important role in the specific response. Interleukin-1 (IL-1) is secreted by activated fish macrophages (Ellsaesser, 1989) which in turn may stimulate T lymphocytes to secrete interleukin-2 (IL-2). Like mammalian IL-2, it is thought that fish IL-2 can promote the clonal expansion of T cells which is partly responsible for T and B cells co-operation. The lymphocytes are produced in the bone marrow of mammals and then distributed to different parts of the body through the blood and lymph systems. Normally they are found in different lymphoid organs. Functionally lymphocytes are divided into two major classes- T lymphocytes (T cells) and B lymphocytes (B cells). In higher vertebrates, T and B lymphocytes not only show functional differences, but they also show distinct developmental differences, with T cells maturing in the thymus, whilst B cells remain in the bone marrow until after maturation. It has been established that teleost fish lymphocyte populations are analogous in many respects to the T cells and B cells of mammals (Clem *et al.*, 1991). Mammalian B cells express immunoglobins (Ig) on their surface and these act as receptors for antigens, whereas T cells are characterised by the presence of a different type of antigen-specific receptor, the T cell receptor (TCR). Although surface immunoglobulin (sIg) is found on all fish lymphocytes, a portion of cells having only the heavy chain regions of Ig molecules on their surfaces have been extracted from thymocyte membranes of carp, *C. carpio* (Secombes *et al.*, 1983).

It was mentioned above that MHC genes are associated with specific immune system of animal and are involved in presenting antigens to T lymphocytes. Fundamentally, the MHC is involved with the recognition of self and non-self antigen.

Histocompatibility restriction of antigen recognition seems evident in teleost fish and has been reviewed by Stet and Egberts (1991).

In this study non-specific immune responses including different immunological parameters and disease susceptibility of different clonal lines were studied. The clonal lines may be fixed for different alleles and they might show different levels of susceptibility to the challenged pathogen. Therefore, their differential response and crossing may suggest genetic variation between lines and possible mechanisms of disease control.

1.11 Objectives of this study

The genetic variation of specific and non-specific immune responses in different clonal groups of tilapia, *O. niloticus* will be examined. Since MHC and some other genes seem to control the immune response of fish, most of the approaches to be used in this study will be based on an examination of these genes. The MHC genes are inherited in the offspring as a haplotype. Homozygous individuals for MHC loci can therefore be produced by chromosome manipulation techniques. The resulting homozygous individuals will be ideal candidates for analysing the genetic variation of the immune responses between groups of fish, *in vitro* and *in vivo*. Therefore, the following objectives were identified for this study :

1. To produce completely homozygous inbred lines and their clones in tilapia, *O. niloticus* by artificial gynogenesis and androgenesis.

2. To verify the homozygous nature of different lines of tilapia, *O. niloticus* by using multilocus DNA fingerprinting and the isozyme locus *ADA**.

3. To determine the occurrence of major histocompatibility complex in tilapia, *O. niloticus* by PCR and scale grafting.

4. To examine the variation in the non-specific immune responses of clonal groups of tilapia, *O. nioticus*.

5. To establish the susceptibility of clonal lines of tilapia, *O. niloticus* to an infectious pathogen, *Aeromonas hydrophila* and link this to possible genetic differences for non-specific genes.

Chapter II

Production of inbred fish and clones in *Oreochromis niloticus* by gynogenesis and androgenesis

2. Production of inbred fish and clones in *Oreochromis niloticus* by gynogenesis and androgenesis

2.1 Introduction

2.1.1 Gynogenesis

Induced gynogenesis is a process of reproduction in which eggs are fertilised with genetically inert sperms and there is no contribution from the male genome to the development of eggs other than stimulation of development. As a result, the embryonic development takes place with the inheritance of only maternal chromosome set (s). Although the sperm DNA is destroyed by UV irradiation they are still motile and initiate embryonic development of eggs (Stanley and Sneed, 1974; Ijiri and Egami, 1980; Allen, 1987). The haploid embryos possess a single set of maternal chromosomes, show "haploid syndrome" and rarely survive more than 48 hrs post hatching in all species. There is no strong evidence for frequent spontaneous diploidization of eggs fertilised with UV-irradiated sperm although it has been exceptionally observed in plaice, *Pleuronectes platessa* (Thompson *et al.*, 1981) and carp, *C. carpio* (Cherfas *et al.*, 1991).

The haploid chromosome set can be diploidised by applying physical shocks (heat, cold, and pressure) at different developmental stages of the eggs. Early shocks prior to loss of second polar body can cause retention of the second polar body and produce meiotic gynogenetics, while late shocks prevent the first cleavage and produce mitotic gynogenetics (see reviews by Purdom, 1983 and Chourrout, 1987). Since 1960 meiotic gynogenesis has been successfully induced in many fish species such as cyprinid loach (Suzuki *et al.*, 1985); common carp, *C. carpio* (Golovinskaya, 1968; 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, *C. idella* (Stanley and Sneed, 1974; Stanley, 1976); plaice, *P. platessa*, and flounder, *Platichthys flesus* (Purdom, 1969; Purdom *et al.*, 1976; Thompsom *et al.*, 1981); rainbow trout, *O. mykiss* (Chourrout and Quillet, 1982; Thorgaard *et al.*, 1983;

Chourrout, 1984; Lou and Purdom, 1984a; Thompson and Scott, 1984; Kaastrup and Horlyck, 1987); zebrafish, *B. rerio* (Streisinger *et al.*, 1981); Indian major carp, *Catla catla, L. rohita* (John *et al.*, 1984); tilapia, *Oreochromis* spp (Chourrout and Itskovich, 1983; Penman *et al.*, 1987b; Don and Avtalion, 1988b; Hussain *et al.*, 1993); silver barb, *P. gonionotus* (Pongthana *et al.*, 1995). Meiotic gynogenetic animals are partially heterozygous because of recombination between non-sister chromatids during the first meiotic division of eggs (Purdom, 1969; Nace *et al.*, 1970; Hussain *et. al.*, 1994a). Initially it was thought that meiotic gynogenesis might be an effective way to produce homozygous inbred lines (Golovinskaya, 1968 and Purdom, 1969). It was expected that during meiosis the rate of gene-centromere crossing over would be very low and a very high degree of homozygosity would be observed in the first generation of meiotic gynogenetics (Nagy, 1987). However, several studies revealed that this method of inducing inbreeding is not as effective as once thought (Thompson, 1983; Allendorf and Leary, 1984; Nagy and Csanyi, 1984). It has been found that the high rate of recombination for loci distal to the centromere could result in some progeny having 100% heterozygosity at such loci.

However, despite the aforementioned drawbacks of meiotic gynogenesis, it is a very useful tool for estimating gene-centromere recombination rates, rate of inbreeding, the identification of sex determining mechanisms, gene mapping (Thorgaard and Allen, 1987) as well as the production of single sex populations in homogametic fish species. Meiotic gynogenesis can also be used to produce clonal lines from homozygous female individuals. Isogenic lines (by crossing inbred lines) in which every individual has the same genotype but is not necessarily homozygous at every locus, can be produced from meiotic gynogenetics. These lines can be used as internal controls in e.g. growth trials (Mair, 1993).

Due to the persistent heterozygosity in the meiotic gynogenetics it is not possible to produce completely homozygous individuals unless some form of sib mating is used in conjunction with this. Thus researchers wanting to produce homozygous lines have focused their attention on mitotic gynogenesis. In mitotic gynogenesis completely

homozygous individuals can be produced by suppression of the first mitotic division followed by replication of a haploid set of chromosomes. Using this technique it is possible to produce completely homozygous individuals in the first generation and clonal lines in the second generation.

The earliest attempt to induce mitotic diploidization by interfering with first cleavage of eggs in plaice, *P. platessa* was not successful (Purdom, 1969). The first successful mitotic diploidization was reported in zebrafish, *B. rerio* by Streisinger *et al.* (1981). Afterwards this technique became more familiar to others who attempted to produce mitotic gynogenetics using different shock treatments, such as heat shocks (Purdom *et al.*, 1985); late cold shocks (Krasznai and Marian, 1987) and pressure shocks (Chourrout, 1984; Onozato, 1984; Naruse *et al.*, 1985). Successful mitotic gynogenesis has also been reported in a number of fish like common carp, *C. carpio* (Nagy, 1987; Komen *et al.*, 1991); medaka, *O. latipes* (Ijiri, 1987); ayu, *P. altivelis* (Taniguchi *et al.*, 1988); Nile tilapia, *O. niloticus* (Mair *et al.*, 1987; Hussain *et al.*, 1993; Myers *et al.*, 1995) and Asian carp, *L. rohita* (Hussain *et al.*, 1994b).

Mitotic gynogenetics are seen to be a very promising product for aquaculture research and can be used to produce clonal lines. As the suppression of first cleavage of eggs during mitotic gynogenesis facilitated elimination of deleterious alleles from the inbred lines, the successful inbred lines could be crossed to obtain a degree of heterosis for commercially important traits. By this way, a higher yielding and more homogeneous strain could be developed for aquaculture. Taniguchi *et al.* (1988) mentioned that mitotic gynogenesis could be considered more useful than meiotic gynogenesis for fixation and establishment of a new race of fish in aquaculture However, both meiotic and mitotic gynogenetic techniques separately and in combination can result in the production of important information on the genetic analysis of traits and in the production of unique genotypes that could have important research as well as production possibilities.

The induction of gynogenesis has shown the possibility of producing monosex population in fish in which the female parent was homogametic (Golovinskaya, 1969,

Nagy *et al.*, 1978, Refstie *et al.*, 1982). In *O. niloticus* and *O. mossambicus*, gynogens are almost all female which represents normal homogametic XX genotypes of female parent (Penman *et al.*, 1987a; Mair *et al.*, 1991a). In silver barb (*P. gonionotus* Bleeker), the all female progeny in the meiotic gynogenetics suggested their female's homogamety (XX) (Pongthana *et al.*, 1995). Hormonal sex reversal of gynogenetic females to males and use of these males can ensure the production of all female population. When the sex-reversed females (XY) are used for gynogenesis, male progenies of mitogynes will be YY which could be used for the production of all-male progeny for culture (Varadaraj and Pandian, 1989; Mair *et al.*, 1991a). In contrast meiogynes can not effectively be used for sex control experiments as recombination takes place in the sex determining locus in *O. aureus* and *O. niloticus* (Avtalion and Don, 1990; Mair *et al.*, 1991a,b).

2.1.2 Androgenesis

Induced androgenesis is a special kind of reproduction process in which genetically inactivated eggs are fertilised with normal sperms so that no chromosomal contribution from the maternal side is incorporated into the embryo and the resultant embryo develops with entirely paternal chromosome inheritance. Therefore, for diploid androgens the maternal chromosome set needs to be inactivated and the embryo develops with only the paternal chromosome set. The inactivation of the nuclear DNA of the egg can be done by using some form of ionising radiation. Gamma [^{60}Co] and X-ray radiation are most commonly used. Because of their high penetration, large number of eggs can be treated at once (Purdom, 1969; Arai *et al.*, 1979; Parsons and Thorgaard, 1985; Grunina and Nejfakh, 1991; May *et al.*, 1988). On the other hand, ultra-violet light (UV) has been successfully used for irradiation of amphibian eggs (Gurdon, 1960; Gillespie and Armstrong, 1980) and also for fish eggs (Kowtal, 1987; Bongers *et al.*, 1994; Myers *et al.*, 1995).

It has been reported that the induction of androgenetic haploids using gamma radiation has been successful in loach, *M. fossilis* (Romashov and Belyaeva, 1964); flounder, *P. flesus* (Purdom, 1969); amago salmon, *Onchorynchus masou* (Arai *et al.*, 1979); rainbow trout, *O. mykiss* (Parsons and Thorgaard, 1985); and brook trout, *Salvelinus fontinalis* (May *et al.*, 1988). Haploid androgens have also been produced by using UV radiation in Nile tilapia, *O. niloticus* (Myers, *et al.*, 1993) and in common carp, *C. carpio* (Bongers *et al.*, 1993).

Yamazaki (1983) observed the spontaneous occurrence of androgenetic individuals where the oocyte was overripe or in some interspecific or intergeneric crosses in which the pronuclei did not fuse.

The first successful production of androgenetic diploids was reported by Gillespie and Armstrong (1980, 1981) in the Mexican axolotl using heat shock (36-37 ^{o}C for 10 mins) or hydrostatic pressure (14,000 p.s.i. for 8 mins) at 5.5 hrs post fertilisation. Androgenetic diploids were first produced in salmonids by suppression of first mitotic division using pressure shock (8,500 - 9,000 p.s.i. for 3 mins) (Parsons and Thorgaard, 1985; Scheerer *et al.*, 1986; May *et al.*, 1988; Nagoya *et al.*, 1996) and later by heat shock at 29 ^{o}C for 10 mins (Thorgaard *et al.*, 1990). Recently, androgenetic diploidization was induced in Nile tilapia (*O. niloticus*) using UV (150 $\mu W\ cm^{-2}$ for 2 to 10 mins) inactivation of the eggs' nucleus and late heat shock at 41-42 ^{o}C for 3 to 6 mins at 22.5-37.5 mins post fertilisation (Myers *et al.*, 1995).

Androgenesis is a very useful technique for the production of homozygous inbred lines. Its most important potential application is to recover the genotypes from cryopreserved sperms, particularly for those which are facing extinction because of environmental or other pressures (Stoss, 1983). Androgenesis can also be used to study the phenotypic effects of cytoplasmic constituents such as mitochondria as was pointed out by Thorgaard (1986). It can be used to analyse sex determining mechanism in fish. Production of homozygous inbred lines by androgenesis could be extremely useful for monosex culture as the genotypes of inbred androgens in *O. niloticus* would be either

YY or XX and any cross generation would be XY males. Hormonal sex reversed androgenetic females (YY) can also be used to produce all male offspring.

Although androgenesis has a number of potential applications in aquaculture, it is very difficult to produce viable diploid androgens due to their high mortality. This high mortality of androgens may result from the damage of the egg due to irradiation for inactivation of its nuclear DNA and shock treatment for inhibition of the first mitotic cleavage.

2.1.3 Clones and their importance

The term "clone" means a group of individuals which are genetically identical, that is, there is no genetic difference among them. The second generation of mitotic gynogens or androgens are clones, so that the resultant offspring would be homozygous for every gene locus.

Both mitotic gynogenesis and androgenesis can be applied to produce completely homozygous individuals in the first generation and clones in the second generation. The inhibition of first mitotic division of eggs using heat shock can produce homozygous fish through both types of artificial reproduction (Mair, *et al.*, 1987; Hussain *et al.*, 1993; Myers *et al.*, 1995). Clones can be produced from mitotic females by applying meiotic gynogenesis and from androgenetic males by another round of androgenesis. Along with mitotic females homozygous mitotic males may produce in the F1 generation and clones can be produced from these males by androgenesis. Likewise, homozygous androgenetic females are produced in the F1 generation and clones can be produced from these females by meiotic gynogenesis. Since clones are completely homozygous for every gene locus, they have potential for fixing superior genes (e.g. disease resistant genes). Thus a pure "gene pool" can be built up in a clonal line which might be similar to a starting population. Clonal lines are supposed to be free from recessive lethal and major deleterious alleles. They can be used in selective breeding programmes for improving fish stocks (Han *et al.*, 1991).

The production of clones of fish is of great importance in aquaculture research. They can potentially be used in a variety of research areas, including immunology, endocrinology, toxicology, sex determination and quantitative genetics. As clones are fully homozygous animals, any interesting genes and their functional expression can be examined in them. They are the identical offspring of homozygous mother or father, so sex ratio of clones might be used to reveal the sex determining factors of a given species. Clones are pure animals and are supposed to response to any kind of adverse environmental changes, therefore they can be used as test animals in toxicological bioassays. Due to the versatility of their application, a number of research groups have attempted to establish clonal lines for a variety of fish species. So far, clones have been successfully produced for zebrafish (Streisinger *et al.*, 1981), medaka (Naruse *et al.*, 1985; Ijiri, 1987), common carp (Komen *et al.*, 1991), ayu (Han *et al.*, 1991) and Nile tilapia (Hussain, 1992). Recently, androgenetic clones have been successfully produced in amago salmon, *O. masou* (Nagoya *et al.*, 1996) and in Nile tilapia, *O. niloticus* (J.M. Myers, personal communication).

In the present study inbred and clonal lines were produced by applying both gynogenetic and androgenetic techniques. Most of the inbred clonal lines were produced by gynogenesis and these lines were used to examine non-specific and specific immune responses in tilapia. Some gynogenetic cloned females were sex reversed to males by hormone treatment and used to produce outbred clonal lines. The sex determining mechanism of *O. niloticus* was also analysed by using these inbred and outbred clonal lines.

2.1.4 DNA fingerprinting

DNA is a giant and polymeric molecule which is composed of two single strands. Each of them consists of a backbone of sugar and phosphate with a number of bases, adenine (A), cytosine (C), guanine (G) and thymine (T) attached to the backbone. Two single chains of a DNA molecule are held together by forming hydrogen bonds between complementary bases (A=T and C=G) and each pair of complementary bases is called a base pair (bp).

In eukaryotic genomes, highly polymorphic DNA sequences can be repeated several to many times. According to the organisation of repetitive DNA in the genome, they can be divided into two classes, interspersed repeat sequences and tandem repeat sequences. In the interspersed repeats, the repetitive DNA sequences are scattered at multiple sites throughout the genome. In contrast, the tandem repeats consist of arrays of two to several thousand repetitive DNA sequences that are arranged in a head-to-tail fashion. This repetitive DNA sequence in tandem repeats is an integral component of eukaryotic genomes and about one-third of the genome consists of such sequences. Considering the length and copy number of the basic repeated elements, tandem repeats can be subdivided into three groups: satellite, minisatellite and microsatellite. Satellites are composed of very high copy number repetitions of a basic sequence (usually between 1000 and more than 100,000 copies). The length of the repeat unit is usually 100-300 bp. Minisatellites consist of shorter repetitive DNA sequences, usually 10-60 bp, and show a lower degree of repetition at a given locus (Jeffreys *et al.*, 1985). Microsatellites comprise very short (between 1 and 10 bp) repetitive sequences and have been called "simple sequences". Microsatellites have a comparatively low degree of repetition and are randomly dispersed throughout the genome (Litt and Luty, 1989).

The three classes of tandem repeats can be highly variable and their variability is most often due to the occurrence of different repeat numbers in different individuals. This variability in repeat numbers forms allelic variants and for a number of mini- and microsatellites almost every individual is heterozygous. Polymorphism caused by such variability is termed as variable number of tandem repeats (VNTR). Polymorphism due

to VNTR within a particular arrays is thought to be generated by unequal crossovers during meiosis and by the mutational process of slipped strand mispairing during DNA replication (Jarman and Wells, 1989; Avise, 1994).

DNA fingerprinting techniques were developed by Jeffreys *et al.*, (1985) using Southern blot analysis of a hypervariable DNA region. The multilocus DNA probes named 33.6 and 33.15 identified a family of tandem repeated DNA sequences which were characterised by a common GC-rich core sequences derived from an intervening sequence of a human myoglobin gene. The DNA profile can be generated by cleaving total genomic DNA on either side of the minisatellite, not within the repeated sequence using a particular restriction enzyme. The length of the restriction fragment will depend on the number of repeats between sites and generate DNA fragment length polymorphism. The DNA fragments are separated by agarose gel electrophoresis, transferred to a membrane and hybridised with one of the complementary probes. The hybridisation results in a DNA profile which is specific for an individual in most natural populations. The complexity of the DNA profile depends on the number of loci recognised by the hypervariable probe. If the probe hybridises to several loci scattered among the genomic DNA, the resultant profile is called "multilocus fingerprinting". Since spontaneous *de novo* mutation is rare (Jeffreys *et al.*, 1987 and 1988) and bands constructing the fingerprint patterns are inherited in Mendelian fashion, each of the bands in an individual DNA fingerprint profile must originate from either its biological father or mother.

Multilocus DNA fingerprinting is the method of choice in many cases especially for paternity and maternity analysis (Jeffreys *et al.*, 1985; Burke and Bruford, 1987; Burke *et al.*, 1989; Burke *et al.*, 1991). It is also useful in determining the relative values of genetic variability within and between populations (Gilbert, *et al.*, 1990; Reeve *et al.*, 1990). However, there are some technical, theoretical and statistical difficulties of multilocus fingerprinting which make it difficult to analyse or inappropriate in some cases like complex mating systems within and between populations.

However, DNA fingerprinting has been used extensively to produce individual specific markers in forensics (Bär and Hummel, 1991), in linkage analysis (Jeffreys *et al.*, 1986) and in measuring genetic diversity (Reeve *et al.*, 1990). In aquaculture research, DNA fingerprinting can be used as a tool for the identification of individuals, constructing pedigrees and population genetic analysis (Hallerman and Beckman, 1988). It also facilitates estimation of inbreeding rates in commercial broodstocks (Doyle and Talbot, 1986; Eknath and Doyle, 1990) and family identification without using tags especially for very small fish. DNA fingerprinting can also protect "breeders' rights" for hatcheries with superior broodstock by providing effective means of differentiation between hatchery-reared and wild-caught fish

In this study multilocus DNA fingerprinting technique was used to identify gynogenetic, androgenetic and clonal individuals. The fingerprints were produced by hybridising genomic DNA with Jeffreys 33.15 probe which produced specific banding patterns of DNA profiles. The analysis of banding patterns of gynogenetic and androgenetic fish and clonal lines are presented and discussed. In addition to the DNA fingerprinting, the gynogenetic and clonal individuals were also identified by determining their genotypes through analysing isozyme locus. Several polymorphic loci can be used for tilapia identification but the *ADA** locus was analysed in this study.

2.2 Materials and methods

2.2.1 Stock of experimental fish

The tilapia species, *Oreochromis niloticus* L. used in the experiments described in this thesis came from the Tilapia Reference Collection at the Institute of Aquaculture, University of Stirling, Scotland. They were originally collected from a wild population in Lake Manzala, Egypt, in 1979 (McAndrew and Majumdar, 1983). The original fish stock has been properly managed to maintain their genetic variation over the generations. Their overall genetic variation was studied by McAndrew and Majumdar, (1983) and Myers *et al.* (1995).

2.2.1.1 Rearing and stocking facilities

All the rearing and stocking activities were carried out in four recirculating fresh water systems maintained in the various tropical aquarium facilities at the Institute of Aquaculture. The temperature in the aquaria was maintained at 28 ± 1 °C and the photoperiod controlled with 12 h light : 12 h dark. The recirculating water system comprised of several components, header tank, stock tank, bottom settling tank, sump tank, pump, water buffering facility, water heater and biological filter trays. Figure 2.1 represents a tilapia broodstock recirculating water system. Fish were kept in glass aquaria so that females' maturity state can be observed from the outside.

The rearing and stocking of the experimental fish, reported in this thesis, were carried out using three different types of recirculatory systems.

2.2.1.1.1 Early fry rearing system

The early fry rearing system consisted of 2 x180 l header tanks, 4-x180 l bottom settling tanks, a pump tank and a large number of 10 l (water depth 13.5 cm) Perspex rectangular rearing tanks. These tanks were arranged in two rows on a two tier system and each of them has a central 20 mm drainage stand pipe. The warm water came from

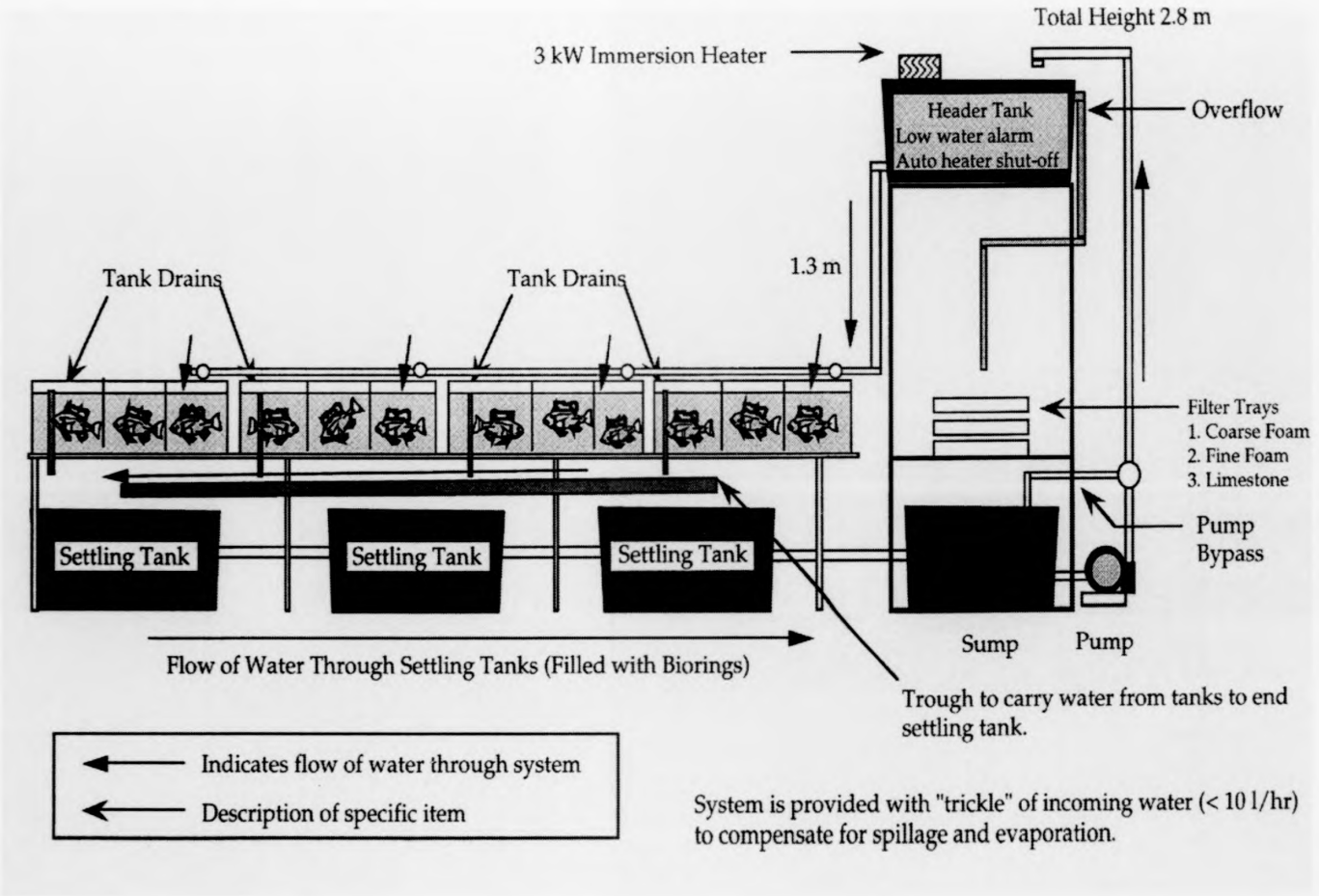


Figure 2.1 Schematic diagram of tilapia broodstock recirculating system (Copied from Mcndrew *et al.*, 1995)

the overhead tanks into the rearing tanks by gravity and the waste along with excess water discharged into the bottom settling tanks through the drainage stand pipe. The top part of the standpipe was covered around with 500 micron nylon mesh screen whichprevented the escape or loss of fry via the overflow. Continuous aeration was provided to the header tank from a central blower unit using one or two 15 cm air stones. The chemical water parameters such as dissolved O_2, pH, NH_3, NO_2 and NO_3 were monitored regularly.

2.2.1.1.2 Advanced fry rearing system

Advanced fry were reared in 30 l (water depth 18.5 cm) circular plastic tanks which were connected with 2 x 180 l header tanks, 2 x 180 l bottom settling tanks and a 180 l pump tank. The circular plastic tanks each having a central stand pipe, were divided into four units, each unit contained 12 tanks arranged in parallel double rows of 24 tanks each side. Each of the tanks was equipped with an inlet having one or two small jets at the blind end and a central drainage stand pipe. When water from the overhead tank came down directly into the circular tank by gravity, a 30^{o} to 45^{o} angled flow was maintained. As a result a circular water flow was created in the tank which facilitated to drain most of the centrally accumulated solid wastes along with excess water through the central outlet pipe into the bottom settling tank.

A series of 180 l capacity settling tanks collect solid wastes. These tanks were equipped with rows of long brushes and/or plenty of floating bio-rings to assist settling and acts as a surface where bio-filtration can take place. The bottom of the settling tanks were cleaned once every three to four weeks by draining out most of the water as well as by flushing of new fresh water. After bio-filtration the water flows into a sump tank from where it was pumped (0.25 H.P., Beresford Pump Ltd.) back to the 2 x 180 l

header tanks. To maintain the pH (6.5 - 7.8) of the recirculating system, the overflowed water from the header tank was passed through trays full of limestone and shell. Sometimes a series of fine synthetic filters were placed on the limestone tank. The water temperature was maintained at 28 ± 1°C by placing a 3 kw heater (Howden Ltd.) into the header tanks.

2.2.1.1.3 Stocking system

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

2.2.1.2 Feeding

Fish of all sizes from fry to brood were fed with an appropriate size (no 2 size to no 5 size) of commercial trout feed (Trouw Aquaculture Nutrition, Rosshive, UK). Although these feeds were not specially formulated for tilapia culture, they were apparently suitable to provide their nutritional requirements. The proximate compositions of different feeds are presented in Table 2.1. The early fry were fed with a micronized food (0.25 - 1.0 mm) prepared by grinding no 3 sized food using a Moulinex coffee grinder. This was fed 3 to 4 times a day *ad libitum*. As the fish grew up the feed size was increased. The advanced fry and fingerlings (10 - 40 g) received no 3 sized food at a rate of 3 - 7.5% body weight 3 times a day. Fish weighing 40 - 80 g were fed with no 4 feed (4 - 4.5 mm) and for 80 + g and broodstock, no 5 feed (5.5 - 6.0 mm) was used, but the ration was reduced to 2-3% body weight.

2.2.2. Anaesthesia

To avoid excess handling stress during breeding, sampling (length and weight measurement), tagging and marking, fin clipping and blood collection, the fish were

Table 2.1 Proximate composition of different feeds used in rearing of experimental fish (% dry matter basis) (Source -Trouw Aquaculture)

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

anaesthetised with benzocaine (ethyl 4-aminobenzoate) at a concentration of 1: 10,000. A stock solution was prepared by dissolving benzocaine powder at 10% w/v in ethanol. Whenever necessary fish were immersed in a bucket containing fresh benzocaine solution and left there until the fish lost equilibrium and opercular movement stopped stopped. In this condition fish could be handled for few minutes while necessary work was done. After handling the fish were moved to the aquarium with rapid water flow and aeration. They usually recovered within a few minutes.

2.2.3 Tagging of fish

Due to a shortage of individual tanks in the aquarium it was necessary to keep fish from different families communally in a large tank. Before mixing they were tagged at 3-4 months old with individual identification marks. For this purpose an electronic PIT tag (Avid Inc. California, USA) was used, which carries an individual nine digit number that can be read by an Avid Tag Reader (Power tracker II, Avid Inc. California, USA). This small tag was placed into the body cavity of the fish in an anaesthetised

condition by making a small dorso-ventral incision just above the anus with a sharp, sterile scalpel blade. After insertion of the tag into the body small amount of Orahesive Protective Powder (E.R. Squibb and Sons Ltd. Middlesex, UK) was spread over the incision to assist healing and prevent infection. Proprietors tagging syringe with a sharp needle was also used to tag some of the fish. The tag was placed into the needle and injected into the body cavity.

2.2.4 Fish breeding

To carry out breeding activities a number of mature female and male brooders were transferred from the brood stock tanks to a series of glass aquarium (120 cm x 44 cm x 30 cm) connected with a recirculating water system as described above (Figure 2.1). The water temperature was maintained at 28±1 °C using a thermostat heater placed into the overhead tank. Usually three females were accommodated in one tank and separated by two sheets of Perspex. All of the brooders were marked with Avid Tags as described above.

Normally mature females of *O. niloticus* spawn at approximately 2-3 week intervals under aquarium conditions. Females which were ready to spawn had a swollen urogenital papilla and showed pre-spawning behaviour such as nest building and cleaning. The eggs were collected from the ripe female by stripping. Fully ovulated eggs are very easily stripped by applying gentle downward pressure with the fingers from below the pectoral fin to the genital opening of the fish. Eggs were collected in a clean, sterile Petri dish (100 mm in diameter) and were washed very carefully with water from the recirculatory system several times until all blood and ovarian fluids were removed. Milt was stripped from male fish in a similar way of egg collection but collected in glass capillary tubes (BDH). Fertilisation of eggs was accomplished *in vitro* by adding the milt to "dry" eggs and then adding 10-20 ml of aquarium water. After fertilisation eggs were transferred to downwelling incubator for further development.

2.2.4.1 Incubation of eggs

A few minutes after fertilisation eggs were washed with aquarium water in the Petri dish and then transferred to a series of 750 ml round bottomed plastic jars (soft drink bottles) for incubation (Figure 2.2). These jars were connected to a recirculating water system where warm water (28±1°C) was fed from a 125 l overhead tank to the jars by gravity. The water from the overhead tank first passed through a 30 W UV sterilisation unit (flow rate 20 l min^{-1}, UV dosage ca 62000 µW sec cm^{-2}), then through20 mm PVC pipe to the jars. They received water from the PVC pipe flow via 4 mm diameter Perspex tubing connection and the flow in the jars was controlled by small airline taps in such a way that the eggs in the jars were kept in gentle motion at all times (Rana, 1986). The wastewater was discharged into the bottom settling tank (180 l) via two filters filled with shell unit positioned just above the settling tank. The shell filters helped to maintain the pH of the system and act as a surface for bacteria.

2.2.5 Application of heat shocks to fertilised eggs

The range of temperatures and duration of heat shocks to fertilised eggs during the artificial induction of gynogenesis and androgenesis in *O. niloticus* are described in details in Sections 2.2.11.1 and 2.2.11.2. Therefore, a detailed description of the equipments and basic methods are given in this section.

A temperature controlled water bath having both cooling and heating facilities with a range of -20 to +100 ± 1 °C (Jencons Scientific Limited, England) was used for heat shock treatments. The water bath was filled with clean water and heated to the required temperature before the experiment was started. A fine mercury thermometer(1-100 °C) with 0.1 °C graduation was also used to check the actual temperature of the water. Eggs were fertilised *in vitro* in a clean Petri dish and then transferred directly to a

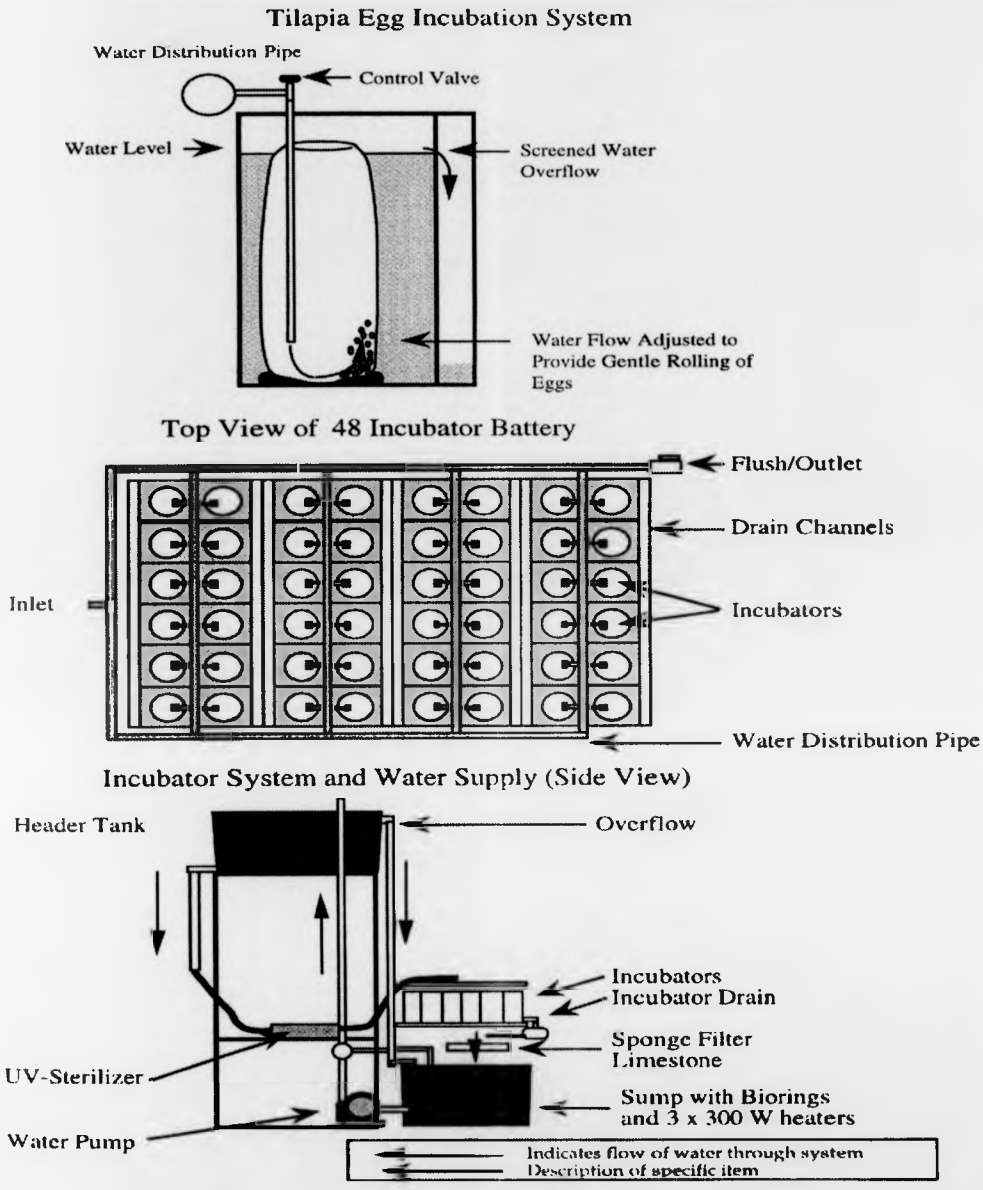


Figure 2.2 Diagram of egg incubation bottles and water recirculating system (Copied from McAndrew *et al.*, 1995)

netting tea strainer placed into the water bath and left for required duration as per the design of the experiment. When the shock period was over, the strainer with eggs moved immediately back to a bucket full of water (28 ± 1 °C) and finally the eggs were moved to the incubator.

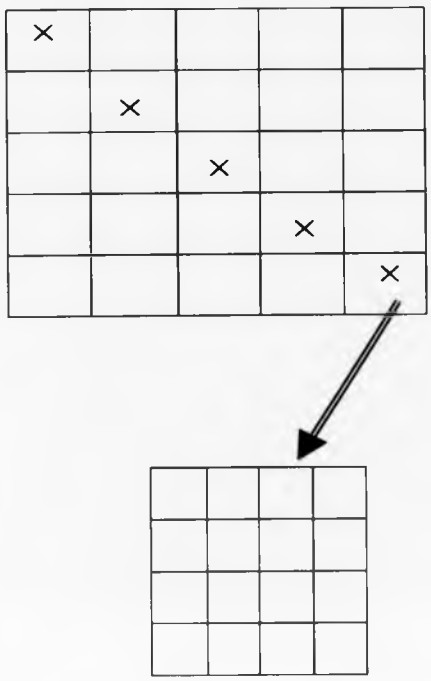
2.2.6 Collection and ultraviolet irradiation of sperms

Collection and UV irradiation of sperm were carried out following the methods described by Hussain (1992) with some minor modifications. Milt was collected from the mature male fish by artificial stripping. Before stripping the urine was ejected and feces or mucus were cleaned from the genital papilla by using a piece of soft tissue. The milt was collected in a glass capillary tube by placing it at the opening of the urethra. Any milt with urine or other contamination were discarded. The milt was always kept in a refrigerator at 4 °C until used in either normal fertilisation or other experimental crosses.

Sperm used for gynogenesis was first checked for motility under a light microscope and then the sperm count was estimated using a haemacytometer. The counting was carried out as follows.

Ten µl of milt was added to 490 µl of Modified Fish Ringers solution (MFR), pH 8.0 (Appendix 2.1), in a microtube to make the total volume 500 µl and mixed by gentle shaking. Then 10 µl of diluted sperm from the first microtube was placed into another microtube containing 90 µl of MFR to give a total volume of 100 µl. The sperm was mixed again and about 12 µl of diluted sperm was placed carefully on each side of the haemacytometer under a coverslip.

After a few minutes when the sperms settled down, they were counted in 5 large squares as indicated above on each side of the haemacytometer and the average taken to minimise any errors during counting. The concentration of sperm was calculated as follows:



For example :

Total number of sperm in 5 large squares on one side was 200 and on the other side was 250

Average number of sperm in 5 large squares = (200 + 250)/2 = 225

Average number of sperm in a small square = 225/80 = 2.81

Total concentration of sperm = $2.81 \times \underset{\text{chamber volume}}{4000} \times 1000 \times \underset{\text{dilution factor}}{50} \times 10 = 5.6 \times 10^{9}\ ml^{-1}$

For UV irradiation the number of sperm was adjusted to $2.5 \times 10^{7}\ ml^{-1}$ by diluting $(2.5 \times 10^{7})/(5.6 \times 10^{9}) = 4.46\ \mu l$ of dry sperm with MFR to give the total volume 1000 µl.

The diluted milt was transferred to a 30 mm Petri dish for irradiation where the depth of the solution was approximately 1.2 mm. The sperm were gently stirred and irradiation was carried out at 4 °C temperature using a 254 nm wavelength UV lamp (Ultra-Violet Products, San Gabriel, Calf.) at a dose of 250 - 265 μW cm^{-2} for 2 mins. The intensity of the UV radiation was measured by a radiometer (Ultra-Violet Products Inc.).

2.2.7 UV irradiation of eggs

UV irradiation of eggs was carried out according to Myers *et al.* (1995). The UV irradiation required 4 ml of unfertilised eggs, which were measured in a graduated vial. The eggs were washed in water by very gently inverting the tube a few times. The wash water was poured off and new fresh water added to bring the total volume of eggs and water to 14 ml. The eggs in water were then poured into a Petri dish (75 mm in diameter) which was then placed on an egg "whirler" under a 254-nm UV lamp (Ultra-Violet Products, San Gabriel, Calif.). The egg's DNA was denatured by UV irradiation with a dose of 150 μW cm^{-2} for 4 mins. The intensity of UV light was measured by a radiometer (Ultra-Violet Products, San Gabriel, Calif.).

2.2.8 Karyological study for ploidy determination

For ploidy determination of the experimental fish metaphase chromosome spreads were prepared from newly hatched or one day old larvae following some minor modifications of the original procedures described by Kligerman and Bloom (1977), Chourrout and Itzkovich (1983) and Chourrout (1986). Embryos were transferred to a Petri dish containing 0.002 - 0.005% colchicine solution and left for 4-6 hrs at 25 °C. After that they were transferred to a chilled 0.7% NaCl solution and the head and yolk sac removed under a binocular microscope using a pair of surgical needles. The dissected tissues were kept in distilled water (hypotonic solution) for 8-12 mins before fixed in 3 : 1 methanol : acetic acid. The embryonic tissues were left in the fixative for

about 30 mins but, if necessary, they could be stored for up to 6 weeks at 4 °C providing the old fixative was replaced twice with fresh.

For slide preparation the embryonic tissues were removed from the fixative, and excessive fixative blotted off with tissue. They were then placed in a 5 mm diameter and about 7 mm deep flat-bottomed hole (made in a 10 mm thick Perspex block) with 2-3 drops of 50% acetic acid. The tissues were then ground for 1 min using a 3 mm diameter glass rod. Ten mins after grinding the cell suspension was taken up into a capillary tube and dropped from 30-40 cm height on to a clean glass slide placed on a hot plate (45 °C). To make a fine circle from the drop most of the remaining fluid was sucked back into the capillary tube within 8-10 sec and the process repeated to produce 2 or 3 rings per slide.

Slides were removed from the hotplate after 1 min and left to air dry before staining with 10% Giemsa (prepared in 0.01M phosphate buffer, pH 7.0) for 20 mins. The slides were then rinsed in distilled water to remove excess stain, air dried again, placed in xylene for 10 mins and finally mounted with DPX (BDH Ltd.). Chromosome spreads were identified around the edge of the circle under x40 magnification and the number of chromosomes counted under x100 (oil immersion) magnification using an Olympus compound microscope.

2.2.9 Sex reversal

As per the requirement of the experiment sex reversed fish were produced by feeding hormone treated food from first feeding. This occurred approximately 9-10 days after fertilisation, when the yolk sac has just been absorbed completely. Usually half of the fry of a batch were fed with hormone treated food for 30 days followed by untreated food. The remaining half was kept as untreated controls to give the original sex ratio.

17 α-methyl testosterone (60 mg kg^{-1} of food) was the hormone used to produce masculinized females are called "neomales" (fish which are genotypically female but phenotypically male) (Popma and Green, 1990). Sex reversed females ("neofemales")

can be produced by using Diethylstilbestrol (DES) hormone added to the food (Mair and Santiago, 1994).

To prepare the food, the no. 3 trout feed pellets were ground to give a fine powder and then thinly spread out in a tray. The required amount of 17 α-methyl testosterone or DES hormone was weighed and dissolved in ethanol (50 ml of ethanol was used per 100 g of ground food). The dissolved hormone was sprayed over the food in a fume cupboard using a spray gun (BDH) and the food thoroughly mixed. The food was left in the fume cupboard for several hours to dry, then stored in airtight containers at 4 °C. The food for control groups of fry was prepared in the same way by spraying only ethanol.

2.2.10 Fish sexing

For sexing fish two methods are used. The method used to sex mature fish is examination of the morphology of the urogenital papilla. Generally, in the case of mature males, the urogenital papilla possesses a common posterior opening, whereas the female fish has a separate urinary and an oviducal openings (Chervinski, 1983). This method is very reliable for large fish but in smaller immature fish where the morphology of the urogenital papilla is not so prominent, there is a possibility of misidentification.

The second method used was gonad squashing and aceto-carmine staining (Guerreo and Shelton, 1974). This is used for small immature fish and is more accurate and reliable than morphology. The fish is killed and the viscera removed to reveal the two thread like gonads lying along the upper surface of the body cavity on either side of the kidney. The gonads are removed and placed on a clean glass slide. A few drops of aceto-carmine stain are added and the gonads squashed with a coverslip. The sex of the fish was identified by examining the slides under a simple microscope. Figure 2.3 and Figure 2.4 showing the structure of a testis and an ovary of *O. niloticus* respectively.

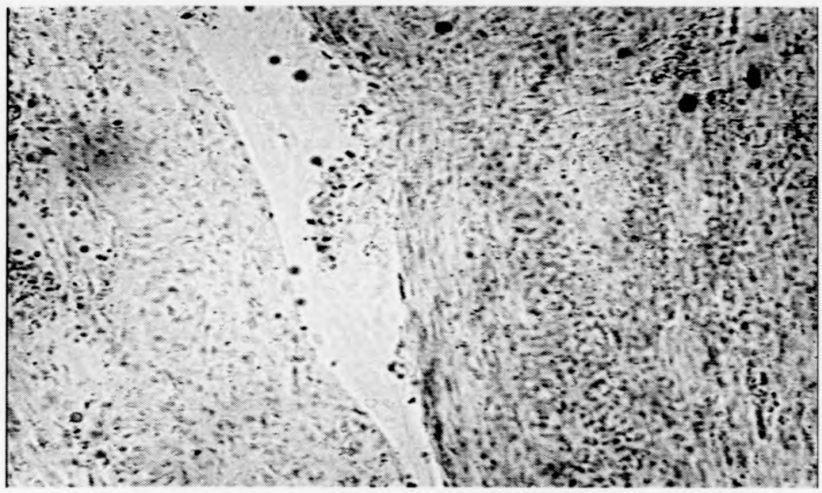


Figure 2.3 Testis of a 12 weeks old *O. niloticus*

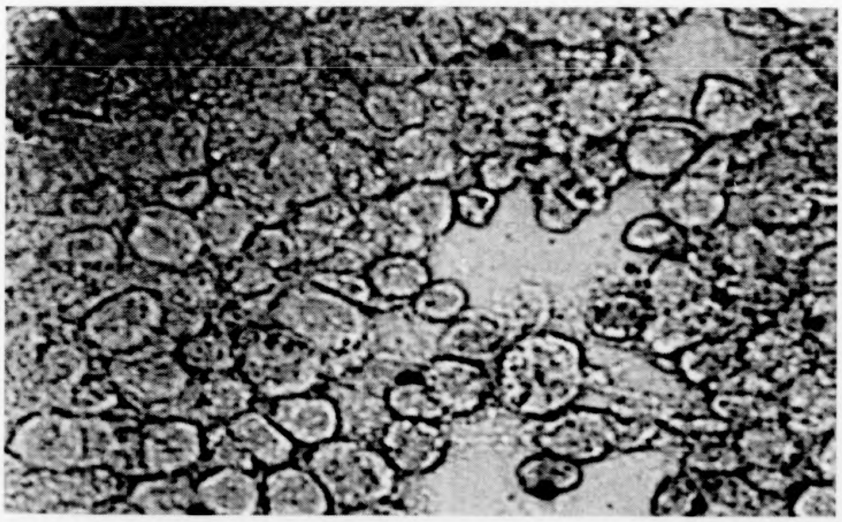


Figure 2.4 Ovary of a 12 weeks old *O. niloticus*

2.2.11 Production of inbred lines

2.2.11.1 Experimental designs for the production of gynogenetic inbred lines

In general this was carried out according to Hussain *et al.* (1993) with some minor modifications. Eggs were collected and fertilised with UV irradiated sperm. To induce meiotic gynogenesis, eggs were heat shocked at 41-42 ^{o}C for 4 mins, starting at 5 mins after fertilisation. The heat shock was applied during second meiosis to prevent extrusion of second polar body. For mitotic gynogenesis, the first cleavage of eggs was suppressed by administering heat treatment at 42-42.5 ^{o}C for 4 mins commencing 25-29 mins after fertilisation. Haploid gynogenetics were produced by fertilising eggs with irradiated sperm and incubated without applying any heat shock. Control groups were generated by ordinary fertilisation. Figure 2.5 shows the schematic diagram of the gynogenetic clonal line production system.

Nineteen mature females of known *ADA** genotype were employed to produce inbred lines. Eggs were collected by stripping from a fully ovulated female (Section 2.2.4) and were divided into four batches. The first, second and third batches of eggs were fertilised with UV irradiated milt (Section 2.2.6) and the fourth batch of eggs fertilised with normal milt. The milt from a single male was used for all egg batches in a single pair mating. The first and second batches of eggs were used for production of meiotic and mitotic gynogenetics by applying early heat shock (41-42 ^{o}C for 4 mins at 5 mins post fertilisation) and late heat shock (42.0-42.5 ^{o}C for 4 mins at 25-29 mins post fertilisation) respectively. The third and fourth batches of eggs were used as UV control and control respectively. All the batches of eggs were fertilised separately with the respective milt. As the meiotic diploidization was carried out 5 mins after fertilisation, the fertilised eggs could be left on the working bench (28 ^{o}C) until the time for the heat shock and transferred into the egg incubator immediately after the shock treatment. The other batches of eggs had to be placed into the incubators just after fertilisation so as they continued to develop at the correct rate (Section 2.2.4.1). The batch of eggs assigned for mitotic diploidization was removed from the incubator just before the time for heat shock (25-29 mins after fertilisation) and put back once they were treated.

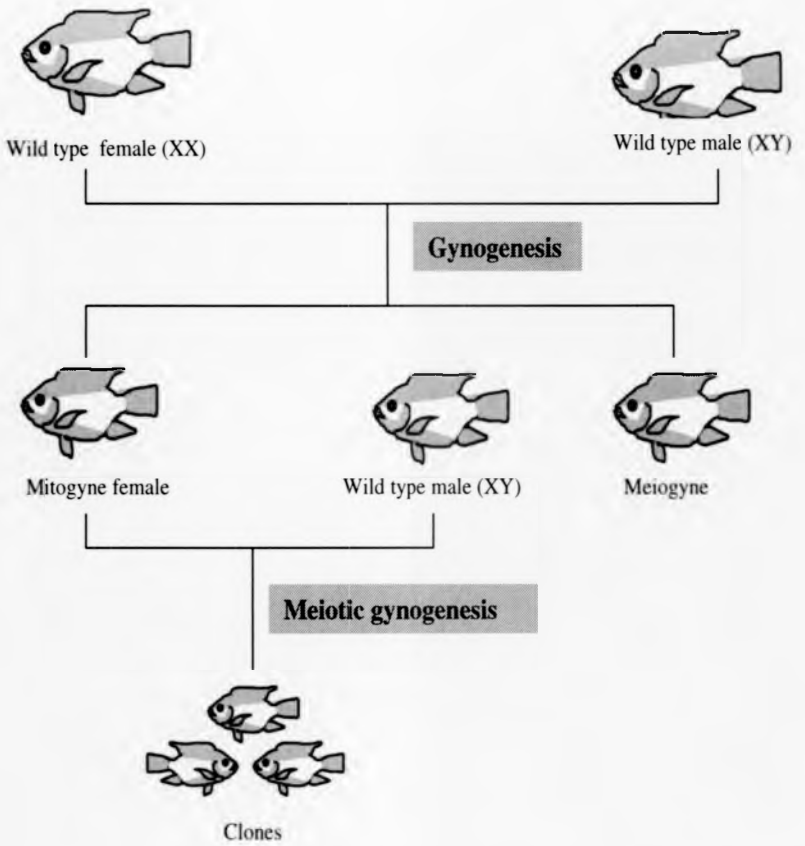


Figure 2.5 A schematic diagram of the gynogenetic clonal line production system in *O. niloticus*

2.2.11.2 Experimental designs for production of androgenetic inbred lines

Androgenesis was carried out by following the procedures described by Myers *et al.* (1995). Before fertilisation of eggs with normal milt, the egg's DNA was denatured by UV irradiation, with a dose of 150 $\mu W\ cm^{-2}$ for 4 mins (see Section 2.2.7). To diploidize the haploid genomes, the first mitotic division of the eggs was suppressed by applying heat shock at 42.5 °C for 4 mins at 25-27 mins after fertilisation.

Androgenetic haploids were produced by fertilising DNA disrupted eggs with normal sperm and incubating them without applying any heat shock. Diploid controls were produced by normal fertilisation of eggs with sperm.

Mature homozygous blond males were selected and used for the production of androgenetic fish. Blond is a recessive colour trait which will only be expressed if the androgenetic treatment has been successful (Scott *et al.*, 1987; Myers *et al.*, 1995). Milt was collected from a homozygous blond male and kept at 4 °C. Eggs were collected from an individual female (Section 2.2.4) and divided into three batches. The first and second batches of eggs were irradiated by UV light and the third batch of egg left as the control. All three batches of eggs were fertilised with the same milt. The first batch of eggs were assigned for the production of diploid androgens, and the second and third batches of eggs for UV control and control respectively. After fertilisation all batches of eggs were placed into separate incubators. The first batch of eggs were removed at the appropriate time for heat treatment and then returned after being treated.

2.2.12 Production of clonal lines

2.2.12.1 Designs for the production of clonal lines

Gynogenetic and androgenetic clones were produced from gynogenetic and androgenetic inbred fish respectively. For gynogenetic clones, eggs were collected by stripping from mature mitotic females (Section 2.2.4) and fertilised with UV irradiated sperm (Section 2.2.6). Five minutes after fertilisation, the eggs were heat shocked at 41-42 °C for 4 mins (meiotic gynogenesis) and placed into an incubator (Section 2.2.4.1).

As the mitotic gynogenetic females are fully inbred some of them do not produce good quality eggs. So, at least five mitotic females from each family were kept in breeding aquaria in order to produce the clonal lines. Eggs were collected from an individual female and divided into three batches. The first and second batches of eggs were fertilised with UV irradiated sperm and the third batch with normal sperm. The first batch of eggs was used to produce the clonal fish by applying early heat shock as described above. The second and third batches of eggs were used as UV control and control respectively.

For the production of an androgenetic clone, eggs were collected from an ordinary mature female (Section 2.2.4) and denucleated by UV irradiation as described in Section 2.2.7. The milt was collected from an inbred androgenetic male (YY) and used to fertilise the denatured eggs. For diploidization, the fertilised eggs were then exposed to heat shock as described in Section 2.2.11.2.

Most of the androgenetic males were able to produce active sperm. The designs of the experiments for androgenetic clone production were exactly the same as the designs followed for androgenetic inbred line production (Section 2.2 11.2) except the sperm donor was an androgenetic male (YY) instead of an ordinary blond male.

2.2.12.2 Production of gynogenetic homozygous and heterozygous clones

Homozygous clones are a group of individuals which are produced in a clonal line by either sib mating or back crossing with their mitotic gynogenetic mother, or by another round of meiotic or mitotic gynogenesis. On the other hand, heterozygous clones are a group of individuals produced by crossing between a male from one clonal line and a female from another line. Since all the gynogenetic clones were female (XX), with a few exceptions where some males (XX) were found, some neomales (XX) were produced in each line by administering hormone treatment using 17 α-methyl testosterone with food (Section 2.2.9). Figure 2.6 shows the crossing patterns for the production of gynogenetic homozygous and heterozygous clones.

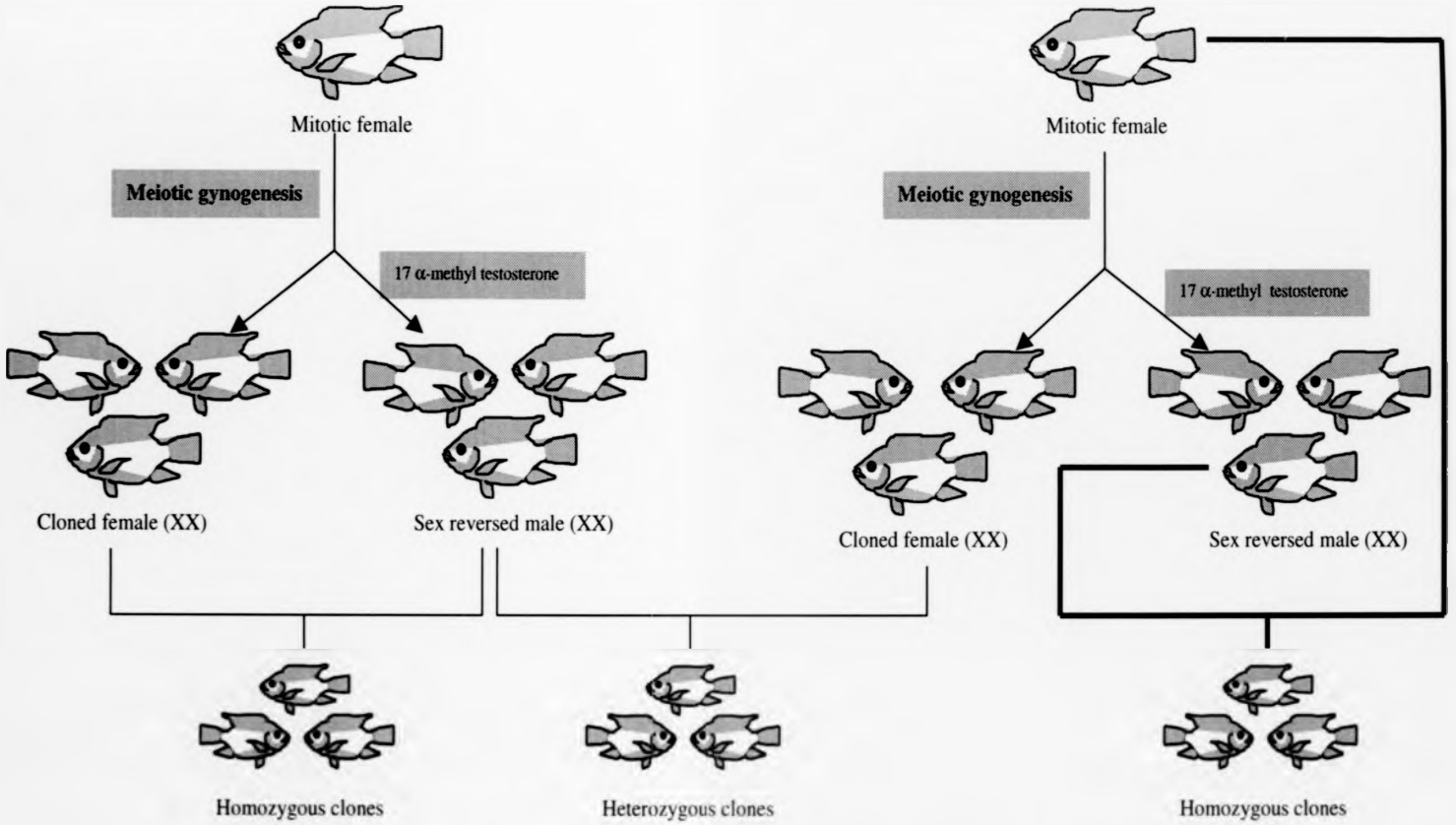


Figure 2.6 A schematic diagram for the production of gynogenetic homozygous and heterozygous clones in *O. niloticus*

2.2.12.3 Production of androgenetic homozygous and heterozygous clones

Homozygous androgenetic clones can be produced either by sibmating or back crossing with their androgenetic father (YY), or by another round of androgenesis (Figure 2.7). Heterozygous clones can be produced by crossing between cloned males (YY) from one line and neofemales (YY) from another line. Neofemales (YY) can be produced by feeding DES hormone with their food (Section 2.2.9).

2.2.12.4 Production of clones from androgenetic female (XX)

In *O. niloticus* male fish are heterogametic (XY) and can produce two different types of gametes. During production of androgenetic diploids both androgenetic male (YY) and female (XX) progeny were obtained. Clones were produced by fertilising eggs collected from the androgenetic female (XX) fish with UV irradiated sperm followed by applying meiotic gynogenesis (Figure 2.7). Some neomales (XX) were also produced by using hormone treatment (Section 2.2.9).

2.2.13 Incubation of eggs and checking their survival rates

Both treated and untreated eggs for all the experiments described in this chapter were incubated identically (Section 2.2.4.1). The embryos in each batch were checked and counted at the pigmentation (40-42 hrs after fertilisation) and yolk sac resorption (9-10 days after fertilisation) stages. The survival rate of the embryos was calculated as follows

$$\text{Survival (\%)} = \frac{\text{number of embryos surviving at a given development stage}}{\text{Total number of eggs}} \times 100$$

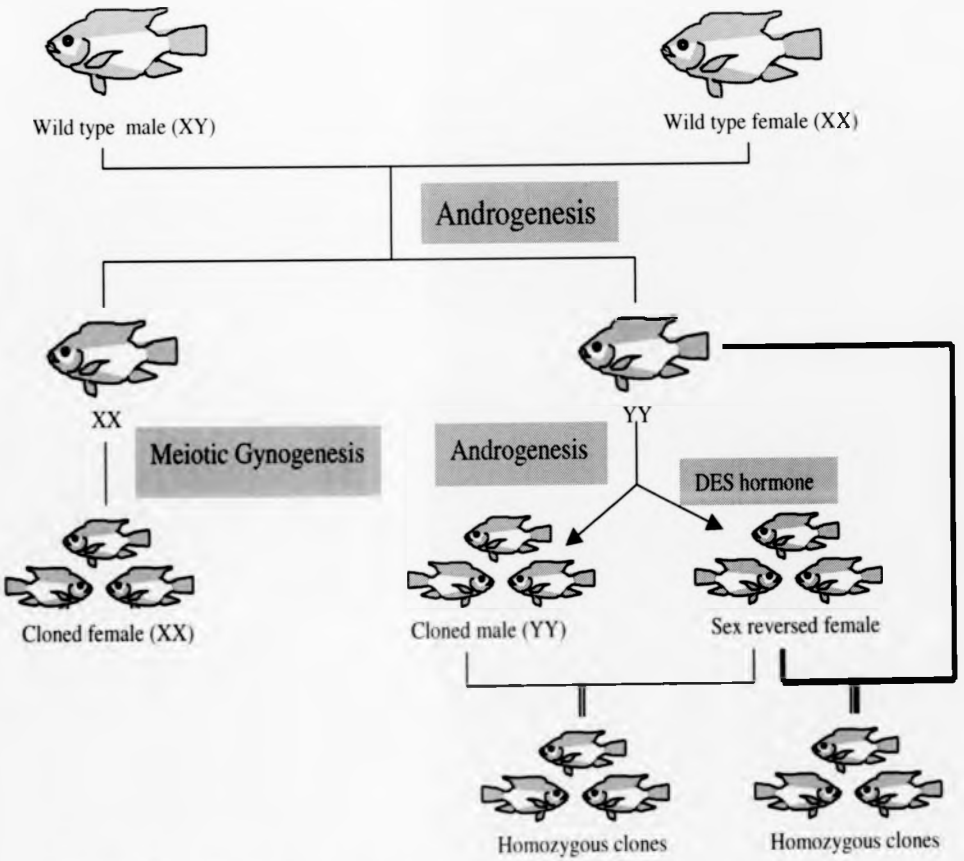


Figure 2.7 A schematic diagram for the production of androgenetic homozygous clones in *O. niloticus*

2.2.14 Progeny testing and determination of sex ratio

The sex ratios in all the treatment groups of fish such as mitotic gynogens, meiotic gynogens, androgens, inbred and outbred clones, and controls were determined by killing the fish, squashing the gonads and staining with aceto-carmine (Section 2.2.10). The fish were sexed at least at a minimum age of three months.

The effectiveness of the hormones and their dosage during the sex reversal experiments were determined by progeny testing. Progeny testing can be used to confirm homozygous condition of the primary sex determining locus in gynogenetic and androgenetic clones. Progeny testing was carried out by either crossing neomales (XX) or androgenetic males (YY) with an ordinary female. Sex ratios of the resultant offspring were determined by killing and sexing them as above. All the sex ratios obtained from different treatment groups and crosses were analysed by Chi-square comparing with 1:1 sex ratio and, if necessary, with the respective normal controls. In χ^2 test both $P<0.05$ and $P<0.01$ were chosen as the significant level.

2.2.15 Starch gel electrophoresis

Horizontal electrophoresis was carried out to verify the genotypes of broods, gynogenetics and cloned progenies produced from different families following the methods described by McAndrew and Majumdar (1983). The total procedures can be divided into three steps : i) Collection and preparation of samples, ii) Preparation of starch gel and iii) Running, slicing and staining of gels.

2.2.15.1 Collection and preparation of samples

a) Fin : Fin samples were collected by simply clipping 3 mm from the caudal fin from different groups of fish using sterile scissors. Samples were put individually into microfuge tubes and stored at -20 °C.

b) Blood : Blood samples were collected from the caudal vein of fish using 21 to 23 g sterile needles and syringes. To prevent coagulation of blood during sampling

Cortland's saline with 10 mM EDTA (Appendix 2.2) (approximately 1 volume of Cortland's saline : 2 volume of blood) was taken in the syringe. The collected blood was mixed with the anti-coagulant by gentle shaking of the syringe and the blood transferred into a 1.5 ml sterile microfuge tube. The blood samples were then centrifuged at 1400 g for 2 mins, the supernatant removed and the pelleted blood cells were stored at -20 °C for future use.

For electrophoresis, samples (fin and blood) were thawed for a few mins. Cytoplasm from the thawed tissues was then absorbed onto 3-4 x 13 mm paper wicks made from Whatman No. 1 filter paper (6 x 6 mm) placed onto the sample in the tubes.

2.2.15.2 Preparation of starch gel

Twenty two g of starch (Sigma Ltd.) was mixed with 220 ml of diluted Tris-Borate-EDTA (TBE) (Appendix 2.3) in an Erlenmeyer flask. The starch was mixed homogeneously and heated up to the boiling point of the solution with continuous rotation of the flask. At boiling point the mixture became a thick gel and the heating was continued until all starch granules had burst and the viscosity began to reduce. Immediately after heating the starch solution was degassed using a water aspirator. The solution was then poured into a 6 mm thick gel former, covered with a glass plate and left overnight for cooling.

2.2.15.3 Running, slicing and staining of gels

After overnight cooling the gel former was removed and a cut was made across gel using a scalpel blade with the help of a ruler, about 3 cm from the edge of one of the long sides of the gel. The filter paper wicks were placed vertically along the cut with a maximum of 30 samples per gel. After placing all the sample wicks, the gel former was placed back on the gel and a Perspex spacer (10 mm) positioned between the gel and the

former to keep the sample slot closed. The gel was then run in an electrophoretic bath with 1 x TBE buffer for 3-4 hrs at 150-200 V at 4 °C in a refrigerator.

Following completion of electrophoresis, the gel was removed from the bath and sliced horizontally into 3 thin slabs. Each slab can be individually stained for a different enzyme, in the present study only Adenosine deaminase (ADA) was examined. For staining the gel, the staining reagents (Appendix 2.3.1) for ADA were mixed with respective buffer and hot agar (50-60 °C) solution. 25 ml of staining mixture were poured over a slab, left for 2-3 mins to set and then incubated at 37 °C until the bands became visible. The stained gel was then fixed in gel fixative solution (Appendix 2.3.2). The visible bands in the gel were examined and scored for the respective genotypes and finally the agar overlay was dried and stored for future reference.

2.2.16 Extraction of total genomic DNA for fingerprinting

2.2.16.1 Sample collection, preparation and digestion

For the extraction of total DNA, fin and blood (both fresh and frozen) samples were used. Both fin and blood samples were collected following the procedures described in Section 2.2.15.1.

For extraction of DNA, fin samples were homogenised in an earthenware mortar and pestle to a fine powder. The tissue was kept frozen all the time during homogenisation by adding liquid N_2. The homogenate was then transferred into a 1.5 ml sterile microfuge tube containing a mixture of 435 µl of TEN buffer (100 mM Tris-HCl, pH 8.0; 10 mM EDTA; 250 mM NaCl), 10 µl of DNAse-free RNAse A (10 mg ml^{-1}) and 50 µl of 10% (w/v) SDS solution. In the case of a blood sample, an aliquot (10 µl) of fresh or thawed blood cells was added to a mixture of 435 µl of TEN buffer and 10 µl of DNAse-free RNAse A (10 mg ml^{-1}) in a 1.5 ml sterile microfuge tube and mixed well. 50 µl of 10% (w/v) SDS solution was then added and the whole mixed

again. After 1 h incubation at 37 °C, 10 µl of proteinase K (10 mg ml^{-1}) was added to each of

the fin or blood digests which were then incubated overnight in the same temperature.

The next day the tubes with digested samples were removed from the incubator and 500 µl of buffered phenol was added to each. The two layers were then mixed by gently shaking and inverting the tubes 10-15 times and the mixture centrifuged at 5000 g for 5 mins in a microcentrifuge (Heraeus Sepatech) at room temperature. The aqueous phase was carefully transferred to another new sterile tube using a micropipette and cut-off sterile pipette tips. This step was repeated another two or three times until the aqueous phase became clear. The solution was then extracted twice with chloroform : isoamylalcohol (24 : 1, vol :vol) following the same procedure as above. The aqueous phase from the last extraction was transferred to a new sterile microfuge tube and 0.6 volumes of isopropanol alcohol were added to the tube and shaken vigorously for a while. At this stage a white precipitate should be seen, otherwise the solution should be kept at -20 °C for 2 hrs (Maniatis *et al.*, 1982) for complete precipitation of the DNA. The precipitate was then pelleted by spinning at 1400 g for 10 mins, the supernatant removed and the pellet washed twice with 70% ethanol, leaving 30-60 mins between washes. The pellet was spun down at 200 g for 2 mins, the supernatant removed and the pellet desiccated *in vacuo* for a few minutes. Finally the pellet was resuspended in 100 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), with 1 h incubation at 37 °C, then stored at 4 °C until further use.

2.2.16.2 Quantitation of DNA

Two methods are widely used for measuring the concentration of DNA in a sample.

2.2.16.2.1 Spectrophotometric determination of DNA

The concentration of DNA of a sample was measured by determining the optical density (OD) at two wavelengths, 260 nm and 280 nm. The OD value at 260 nm is used to estimate the concentration of nucleic acid, while the reading at 280 nm determines the amount of protein in the same sample. An OD of 1 corresponds to approximately 50 $\mu g\ ml^{-1}$ for double-stranded DNA (Maniatis *et al.*, 1982). The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. The ratio for a pure preparation of DNA should be 1.8. If the DNA sample is contaminated with protein or phenol, the OD_{260}/OD_{280} will be significantly less than the above mentioned values. On the other hand, the ratio higher than 2.0 indicates the presence of high amounts of RNA in the sample (Sambrook *et al.*, 1989). For these situations, the samples should be digested again with proteinase K and RNAse A respectively and extracted by phenol and chloroform/isoamyl alcohol (24: 1) following the procedures as described above.

To prepare the sample for spectrophotometric analysis, 5 µl of the extracted DNA was mixed with 995 µl of TE buffer by shaking and then incubating at 37 °C for 1 h. The diluted DNA sample was transferred to a semimicro-UV cuvette (BDH) and 1 ml of TE was placed in another cuvette as a reagent blank. The concentration of DNA was calculated as follows.

$$[DNA] = OD_{260} \times \text{Dilution factor} \times 50$$
$$= X\ \mu g\ ml^{-1}$$

2.2.16.2.2 Mini gel electrophoresis

The concentration of DNA in the samples was also determined by running them in a small agarose gel (0.7%) along with a known standard marker (λ Hind III). For preparing the gel 0.54 g of ultra pure agarose (Gibco BRL) was mixed with 77 ml of 1x TBE (Appendix 2.4) in a 250 ml beaker (Pyrex). The mixture was heated over a Bunsen burner with continuous swirling until no agarose particles were visible. When the gel became cool to 50-60 °C, 3.84 µl of ethidium bromide (10 mg ml^{-1}) was added to the gel and then poured into the gel mould. Usually 1 µg of marker was run parallel to the samples. The gel was run at 3-5 V cm^{-1} for 2.0-2.5 hrs, visualised by an UV transilluminator and photographed by using a Polaroid camera (Polaroid film type 665). For comparing the intensity of a sample band with the marker, one of the marker bands with similar intensity was selected. Then by calculating the proportion of the selected band to the total marker weight the sample DNA concentration was determined.

2.2.17 Digestion of DNA with restriction endonuclease for DNA fingerprinting

Five µg of each DNA sample was digested with HinfI (Stratagene) restriction enzyme in a total volume of 100 µl. The reaction mixture was prepared in a sterile 1.5 ml microcentrifuge tube by adding the required amount of sterile deionised distilled water followed by 10 µl of 10x universal reaction buffer, 10 µl of spermidine trihydrochloride (40 mM), 1 µl of acelytated BSA (10 mg ml^{-1}) and 5 µg of the DNA sample. The reaction mixture was then mixed thoroughly by shaking. Finally the appropriate amount of restriction enzyme (4 unit μg^{-1} of DNA) and one drop of mineral oil were added to the mixture. The tube was then pulse spun at 5000 g to collect the reaction mixture in the bottom of the tube and layer the oil above it. The reaction mixture was then incubated at 37 °C for 14-16 hrs. The mineral oil overlayer on the sample prevented evaporation during incubation. For digestion of multiple DNA samples with the same enzyme, the working conditions can be speeded up by preparing

a premix solution. After aliquoting the premix solution (suppose 50µl) to the individual tubes, the required amount of DNA and restriction enzyme can be added and the total volume raised to 100 µl by adding sterile water.

Following incubation any condensate was accumulated at the bottom of the tube by pulse spinning at 5000 g and 200 µl of TE buffer added to each tube. 300 µl of buffered phenol was added to the tube and it was spun at 8,750 g for 5 mins. The aqueous phase was transferred to another sterile tube and extracted again with an equal volume of chloroform : isoamyl alcohol (24 : 1). At the end of extraction, the aqueous phase was transferred to another tube and mixed with 1/50 volume of 5M NaCl solution. To precipitate the restricted DNA, 2.5 volumes of ice-cold 100% ethanol were added and mixed gently and kept at -20 °C for 2 hrs. Then the tubes were spun at 8,750 g for 15 mins and a white DNA precipitate obtained at the bottom of the tube. The DNA pellet was washed once with 1 ml of 70% ethanol and spun as above. The supernatant was poured off and the DNA pellet dried *in vacuo*. The pellet was resuspended in 6 µl of TE buffer by incubating at 37 °C for 1 h and then stored at 4 °C until needed.

2.2.17.1 Agarose gel electrophoresis

For DNA fingerprinting the restricted DNA was run in an agarose gel. The concentration of the gel depends on the size of the DNA fragments resulting from the restriction endonuclease digestion. Generally, the concentration of agarose gel is increased as the fragment size of interest decreases. 0.7% agarose gel was used for the fragments produced by HinfI enzyme. The gel was prepared by mixing 1.4 g of ultra pure agarose (Gibco BRL) with 200 ml of 1 x TBE buffer in a 500 ml Erlenmeyer flask and the mixture heated with continuous swirling over a Bunsen burner until no agarose

particle could be seen. The gel was left to cool to 50 to 60 °C and then poured into the gel mould. Any air bubbles were removed and a 30 well comb was inserted into the gel.

In about one and half hours, when the gel had completely cooled and solidified, it was placed into a maxi-gel (Pharmacia LKB) bath containing 1 x TBE buffer. 2 l of electrophoresis buffer was enough to cover the gel surface to a depth of 1-2 mm. The comb was carefully removed and ensured that no air bubbles remained in the wells. For gel loading the restricted DNA samples were mixed with 2 µl aliquots of 10x tracking dye (0.1% bromophenol blue, 40% Ficoll) and spun briefly to concentrate the sample at the bottom of the tube. The samples were then loaded into the gel and a suitable size marker (λ-Hind III) also loaded on either side of the gel. After that the gel was run at 1.5 V cm^{-1} for 14-16 h until the bromophenol blue front had migrated down to the end of the gel. This running time allowed all the fragments less than 1.0 kb to migrate out of the gel. At the end of electrophoresis, the gel was stained with 0.5 µg ml^{-1} ethidium bromide for 15-20 mins. The stained gel was placed on a long wavelength UV transilluminator (UVP) and visualised to ensure complete digestion. The marker band positions were measured and the gel photographed using a Polaroid camera.

2.2.17.2 Southern transfer of restricted DNA

Southern transfer of restricted DNA to a non-charged membrane was carried out by using alkaline vacuum blotting (Vacu Gene™, Pharmacia LKB). This blotting system works with a low vacuum pressure during transfer of nucleic acids from agarose gel to a transfer membrane. It is a rapid DNA transfer method and could be completed in a maximum of two hrs.

A 20 x 20 cm non-charged nylon membrane (Hybond-N, Sartorious Ltd), pre-wetted with deionised water, was placed on the pre-wetted porous screen with the shiny

side up. A plastic mask with an opening slightly smaller than the membrane was placed on the membrane in such a way that it overlapped each side of the membrane by approximately 5 mm. Any air bubbles under the membrane were removed. The frame was placed on top of the unit and tightened using the four locking clamps. The gel was then placed on the membrane starting with one of its edges and then gradually sliding it onto the membrane. Any trapped air bubbles were removed carefully by pressing the gel with a gloved finger. It is very important to make sure that the gel and the mask overlapped by at least 2 mm. After placing the gel the pump was turned on and a sufficient amount of 0.2 N HCl solution poured onto the center of the gel just to cover it. This acid depurination step is used to reduce the size of the fragments as small fragment transfer more easily. Besides this, acid/alkaline exposure makes the gel stronger and minimises any collapse during transfer. This acid depurination step should be carried out for about 20-25 mins until the bromophenol blue turned yellow. At this point the entire acid solution is pipetted out and one litre of 0.4 M NaOH solution poured onto the center of the gel and left for 60-75 mins. This denaturation step was employed to produce single stranded DNA that would hybridise with the probe. At the end of the denaturation step, the NaOH solution was poured off, the pump turned off, the wells marked on the membrane and the gel removed. The membrane was then transferred to a tray containing 500 ml of 2 x Standard Saline Citrate (SSC) solution and washed for about 10 mins to eliminate any agarose. The membrane was then air dried for 30 mins, placed between two sheets of 3 mm filter paper and incubated at 80 °C for 2 hrs. Finally the incubated membrane was kept in a clean dry place until needed for hybridisation.

2.2.17.3 Hybridisation of Southern blot membrane with Non-Isotopic Chemiluminescent Enhanced (NICE™) Probe (33.15)

The hybridisation of transferred single stranded DNA fragments with the NICE™ probe 33.15 (Cellmark Diagnostics) was carried out in four steps : pre-washing, pre-hybridisation, hybridisation and post-hybridisation washing.

2.2.17.3.1 Pre-washing

The membrane was wetted with 250 ml of 1 x SSC solution in a tray and then placed into a hybridisation canister with the DNA side inwards. 50 ml of pre-wash solution (0.1 x SSC, 0.5% SDS), pre-warmed at 65 °C, was added to the canister and any trapped air bubbles squeezed out by rolling a pipette along the canister walls. The canister with the membrane was placed in a hybridiser (HB-1, TechNe) and left for 1 h at 65 °C. The purpose of pre-washing is to remove agarose and other particles stuck to the membrane which might be caused unwanted background during hybridisation.

2.2.17.3.2 Pre-hybridisation

Following pre-washing the canister was removed from the hybridiser and the pre-washing solution poured off. 50 ml of pre-hybridisation buffer (990 ml l^{-1} of 0.5 M Na_2HPO_4, pH 7.2 adjusted with concentrated phosphoric acid; 10 ml l^{-1} of 10% SDS) was added to the canister and placed back into the hybridiser. This step lasted for 20 mins at 50 °C.

2.2.17.3.3 Hybridisation

For hybridisation 20 ml of pre-warmed (50 °C) hybridisation buffer (prepared by mixing 900 ml l^{-1} of pre-hybridisation buffer and 100 ml l^{-1} of 10% w/v casein solution)

was prepared in a sterile universal (BDH) and placed in a water bath at 50 °C. Just before replacing the pre-hybridisation buffer by hybridisation buffer, 5 µl of NICE™ probe 33.15 was added to the universal containing hybridisation buffer, mixed homogeneously by inverting the universal several times and then poured into the canister. The hybridisation continued for 20 mins at 50 °C.

2.2.17.3.4 Post-hybridisation washing

Two wash solutions were used. Firstly 50 ml of wash solution 1 (160 ml l^{-1} of 0.5 M Na_2HPO_4, pH 7.2; 10 ml l^{-1} of 10% SDS), pre-warmed at 50 °C, was placed in the canister and the hybridised membrane washed for 10 mins at 50 °C. This was repeated with fresh wash solution 1. At the end of first washing the wash solution 1 was poured off and 50 ml of wash solution 2 (13.8 g l^{-1} of maleic acid, $C_4H_3O_4Na$; 8.7 g l^{-1} of NaCl, pH 7.2) was used for 5 mins at room temperature and was repeated.

The membrane was then removed from the canister and placed with DNA side up on a clean glass plate. Approximately 3-4 ml of Lumi Phos™ 350 (Cellmark Diagnostics) was sprayed over the whole membrane using a spray gun (BDH) in such a way that the membrane did not become oversaturated. The sprayed membrane was then sandwiched between two 21 x 21 cm acetate sheets and any excess Lumi Phos™ 350 squeezed out, avoiding contaminating the outer surface of the acetates.

2.2.17.4 Autoradiography

The sandwiched membrane along with the acetates was trimmed to give an appropriate size for placing it into a 18 x 24 cm light proof Hypercassette. Small pieces of sticky tape were placed along each edge of the membrane to keep the membrane/acetate sandwich secured. A sheet of autoradiography film (Hyperfilm-

MP™, Amersham) was laid on the membrane in a dark room and the cassette closed firmly. The cassette was then kept in an incubator at 30 °C for at least 6 hrs with the first film and then developed. A second exposure to another sheets of film was made for 12 hrs. In the dark room the exposed films were developed with continuous agitation in D19 developer (Kodak) until most of the bands were visible. The film was then transferred to X-ray fixer (Kodak) for 1-2 mins followed by washing with running tap water for 15-20 mins. The autoradiograph was air dried completely and kept in an envelope. As the activity of the chemiluminescence continues for 5 days another film, if required, could be exposed with a longer exposure time.

In most cases 0.7% agarose gel was used to produce DNA fingerprints. Some other higher agarose concentrations were also used to observe variations of resolution of the restricted DNA fragments in different agarose concentrations. Fingerprints produced from 0.7% agarose gel showed that it can make clear resolution of the high molecular weight DNA fragments and provide sufficient information for identifying gynogenetic, androgenetic and clonal fish. It is also very convenient to handle and transfer DNA from 0.7% gel to a membrane. However this concentration can not produce a good resolution of the small DNA fragments. A comparatively clear resolution of the small fragments may be obtained from higher concentrations of agarose (Figure 2.8).

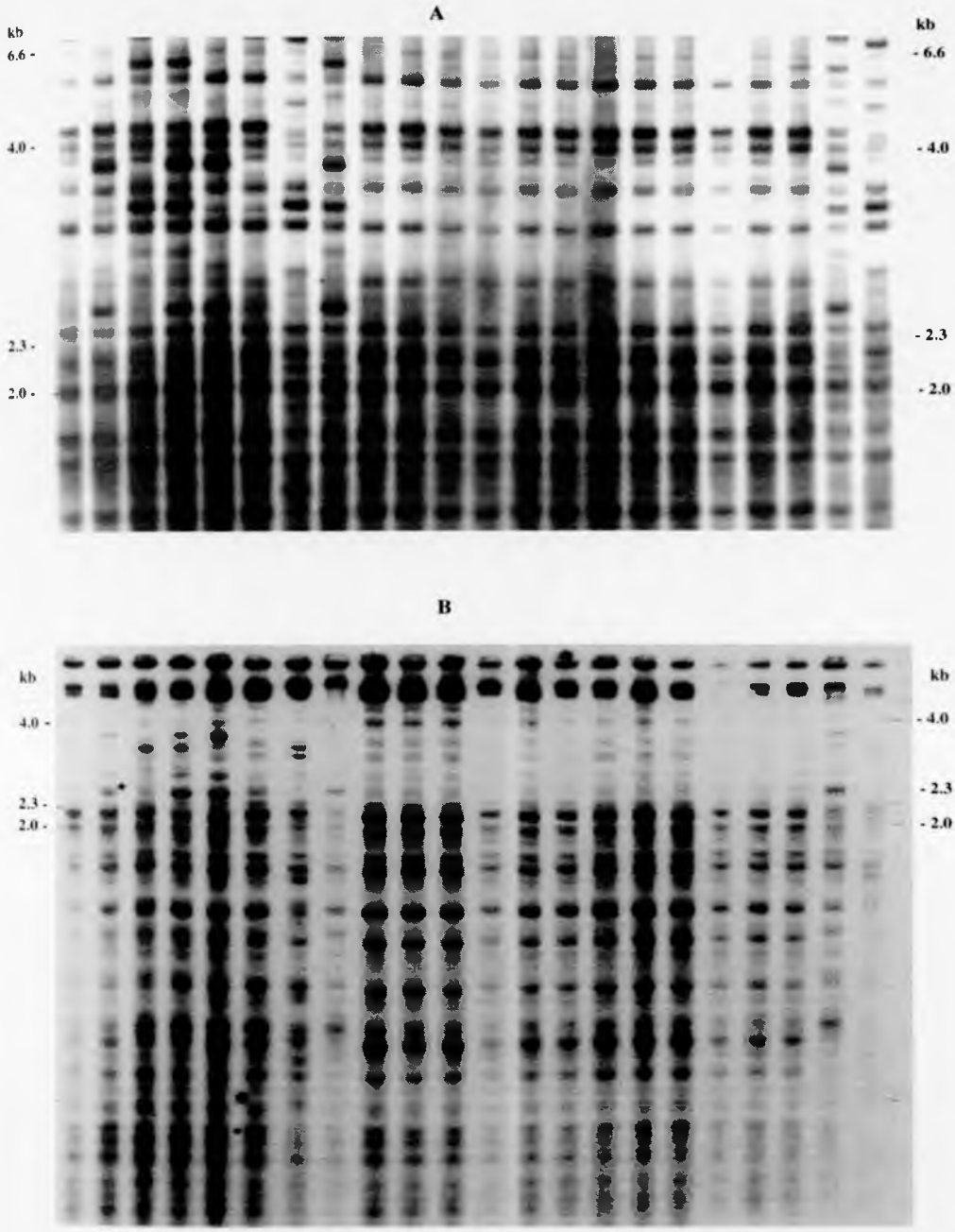


Figure 2.8 Fingerprints showing the differences in resolution of small restricted fragments (less than 4 kb) in two different agarose concentrations. Both fingerprints produced from the same group of individuals by using same restriction enzyme (HinfI) and DNA probe (33.15)
A Fingerprint produced from a 0.7% agarose gel
B Fingerprint produced from a 2% agarose gel

2.3 Results

2.3.1 Production of gynogenetic inbred lines

Figure 2.9 diagrammatically shows the female broods which were involved in the production of gynogenetic clones. In the production of meiotic and mitotic gynogenetics, the inactivation of sperm by UV irradiation was successful. The intensity of UV light used completely destroyed the sperm DNA. All UV control batches showed a sharp reduction in embryonic survival from the pigmentation stage and they were usually all dead in the period between hatching and yolk-sac resorption. Haploid fry display a typical syndrome, a twisted body with an enlarged pericardium. In contrast, the diploid control batches of eggs always showed high percentage of survival and only very rarely had deformities. The chromosome study of haploids, diploid gynogens and controls also confirmed successful UV irradiation of sperm. A single set (n=22) and double sets (n=44) of chromosomes were found in haploid and diploid embryos respectively (Figure 2.10 and Figure 2.11).

The use of heat shock at the different developmental stages of eggs to induce diploid gynogenesis was successful. The karyological analysis of meiotic and mitotic gynogenetics (Figure 2.11) showed an appropriate treatment applied for retention of the second polar body in meiotic gynogenetics and for suppression of first cleavage in mitotic gynogenetics.

The survival percentages at pigmentation and yolk-sac resorption stages of normal controls, meiotic and mitotic gynogenetics are presented in Table 2.2. The survival rate of controls, meiogynes and mitogynes at pigmentation stage showed much difference from their respective yolk-sac resorption stage. Even between the experiments a wide range in survival for all the groups in both pigmentation and yolk-sac resorption stages was found. The survival rates of meiogynes and mitogynes at both pigmentation and yolk-sac resorption stages were significantly lower ($P<0.05$) than controls. A significant drop in survivals between pigmentation and yolk-sac resorption stages were found in all fish groups by an overall survival control>meiogyne>mitogyne. This survival variation might be due to quality of eggs produced by different females

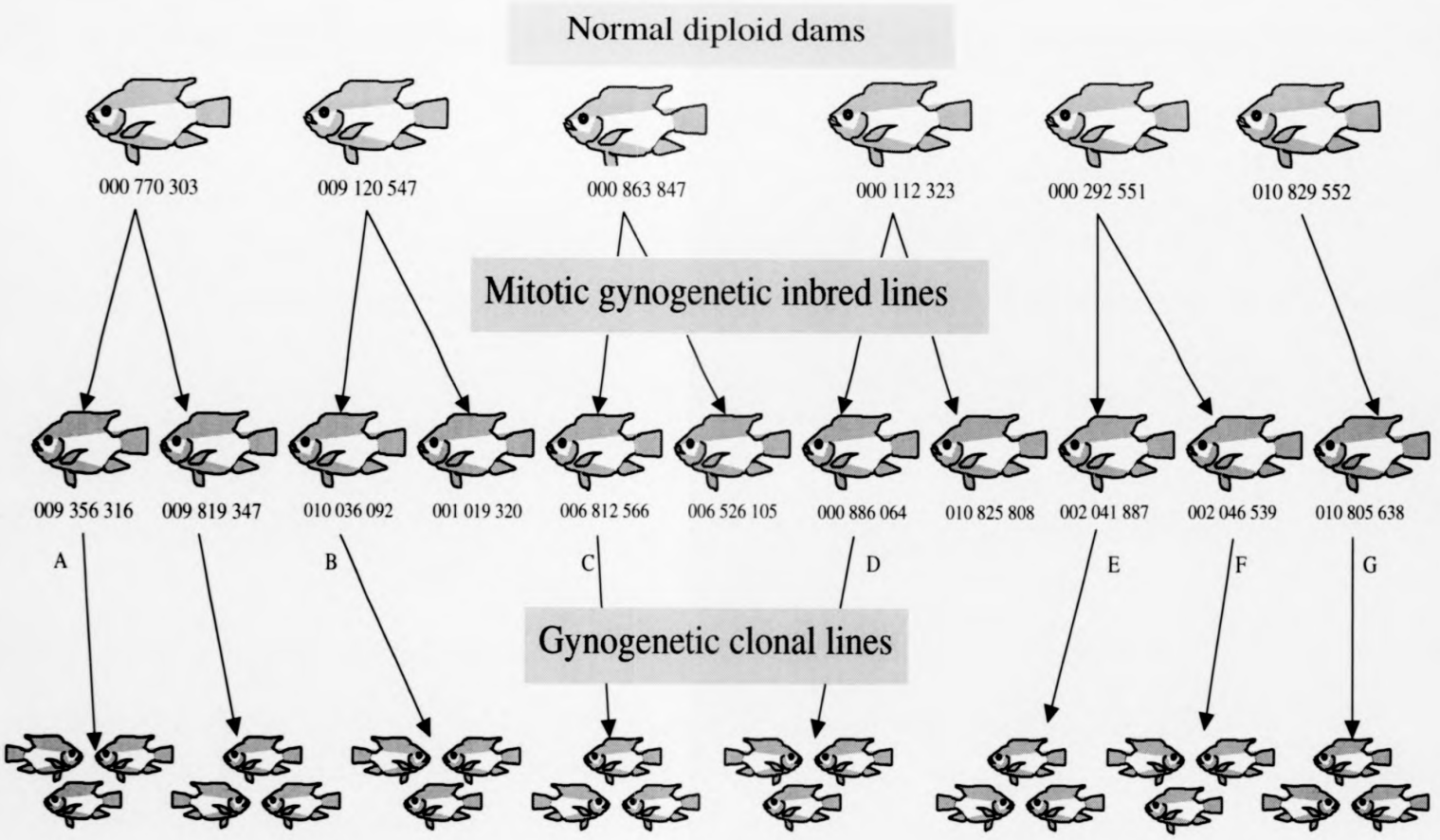


Figure 2.9 Diagrammatic presentation of female broods involved in the production system of gynogenetic clones in *O. niloticus*. Letters A-G used to indicate the name of inbred clonal lines

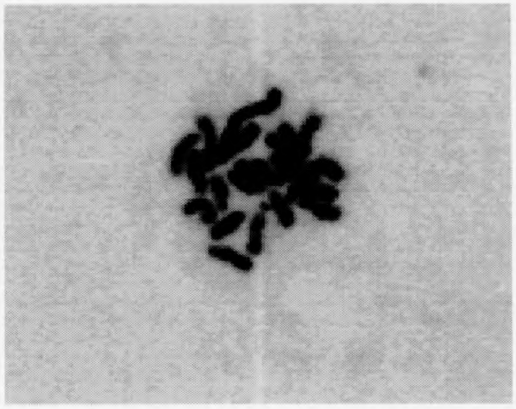


Figure 2.10 Metaphase chromosome spread of haploid (n=22) embryo in *O. niloticus*

Figure 2.11 Metaphase chromosome spread of gynogenetic diploid (n=44) embryo in *O. niloticus*

Table 2.2 Survivals (%) of controls, meiogynes and mitogynes of *O. niloticus* at pigmentation (P) and yolk-sac resorption (YSR) stages in different experiments

No. of expt.	Ordinary ♀	Control		Meiogyne		Mitogyne	
		P	YSR	P	YSR	P	YSR
1	010 829 552[a]	90.0	77.27	73.83	53.02	20.61	9.16
2	000 005 592	92.78	53.61	56.62	17.65	60.0	2.86
3	000 113 623	59.03	50.0	24.14	9.77	4.42	1.26
4	009 120 547[b]	90.16	79.37	60.94	18.75	7.13	0.76
5	000 770 303[c]	91.67	72.22	81.16	55.07	29.29	10.24
6	000 112 323[d]	89.26	68.46	76.67	52.5	28.64	5.10
7	000 863 347	90.09	77.48	41.03	26.92	22.10	7.26
8	000 292 557	54.76	19.84	28.92	8.43	3.21	0.96
9	000 367 361	86.55	69.75	66.67	35.90	19.07	1.48
Mean ± S.E		82.70 ± 4.92*	63.11 ± 6.41**	56.66 ± 6.96*	30.89 ± 6.30**	21.61 ± 5.81*	4.34 ± 1.24**
Mean (RTC) ± S.E.				68.51 ± 5.92	48.95 ± 7.35	26.13 ± 6.06	6.88 ± 1.53

Superscript letters indicate mothers of mitotic females with identical superscript letters in Table 2.3.
RTC- relative to control
The control, meiogyne and mitogyne progenies were produced from all females in a single breeding.
* Significant at P<0.05
** Significant at P<0.05

which also depends on ovulation, physiological condition of fish and system water quality as well.

2.3.2 Production of inbred clones

In order to fulfil one of the main objectives of this thesis, inbred clones were produced from mature mitotic females which were different from each other for major histocompatibility complex (MHC) genes. The procedures for determining MHC genotypes in mitogyne females are described in Chapter 3.

Thirteen inbred clonal lines were produced. Some of them were sibling clones, that is, clonal lines were produced from two or more sister mitogynes (Figure 2.9). Data on survival rates at pigmentation and yolk-sac resorption stages from different clone

production experiments are presented in Table 2.3. The mean survival rates of inbred clones at the pigmentation and yolk-sac absorption stages were 28.67 ± 5.15% and 9.62 ± 2.77% respectively but there was wide variation in survival rates of clones in both development stages between the experiments. The mean survival rates of clones at both pigmentation and yolk-sac resorption stages were significantly different (P<0.05) from controls.

Table 2.3 Survivals (%) of controls and gynogenetic inbred clones of *O. niloticus* at pigmentation (P) and yolk-sac resorption (YSR) stages in different experiments

Mitotic females tag no.	Control		Clone	
	P	YSR	P	YSR
010 561 549[a]	60.19	10.19	23.18	0.52
010 805 638[a]	31.56 ± 0.19	14.94 ± 2.52	10.98 ± 1.58	1.87 ± 0.45
010 036 092[b]	70.15 ± 8.11	42.49 ± 10.40	43.01 ± 11.07	8.01 ± 4.38
001 019 320[b]	14.4 ± 1.31	8.65 ± 0.86	7.32 ± 1.10	1.41 ± 0.69
009 356 316[c]	95.26 ± 2.37	68.39 ± 3.21	19.58 ± 8.98	3.97 ± 2.29
009 864 016[c]	36.88 ± 8.94	23.86 ± 8.66	7.49 ± 5.30	2.11 ± 0.81
009 819 347[c]	81.88 ± 3.86	53.79 ± 6.53	59.94 ± 4.65	21.02 ± 2.54
009 380 256[c]	40.93	31.16	39.81	10.26
009 823 379[c]	72.55 ± 2.45	44.9 ± 2.60	36.12 ± 3.09	23.53 ± 3.15
001 354 550[c]	78.26	49.13	63.65	32.53
000 886 064[d]	61.79	24.39	17.0	4.19
002 046 539	66.50 ± 15.22	44.60 ± 16.39	24.79 ± 18.42	10.32 ± 2.75
002 041 887	83.92 ± 0.22	64.65 ± 2.95	19.90 ± 1.12	5.36 ± 1.69
Mean ± S.E.	61.10 ± 6.56*	37.01 ± 5.49**	28.67 ± 5.15*	9.62 ± 2.77**
Mean (RTC) ± S.E.			46.92 ± 6.83	25.99 ± 5.26

Superscript letters indicate mitotic females produced from normal females with identical superscript letters in Table 2.2

* Significant at P<0.05
** Significant at P<0.05

2.3.3 Verification of inbred and clonal lines by allozyme and DNA fingerprinting

In meiotic gynogenetics the retention of the second polar body using early heat shock produces a proportion of heterozygous diploids depending on the rate of recombination between non-sister chromatids during the first meiotic division (Hussain *et al.*, 1994a; Purdom, 1969; Nace *et al.*, 1970). Genes distal to the centromere are much more likely to remain heterozygous. In contrast, the late heat shock used for suppression of first cleavage produces fully homozygous diploids through replication of a haploid chromosome set.

All of the methods used for verification confirmed success in the production of meiotic gynogenetics, mitotic gynogenetics, androgens and their clones. The occurrence of heterozygosity in meiogynes and homozygosity in mitogynes was observed when they were screened by the isozyme locus *ADA** and by multilocus DNA fingerprinting. The genotypes at the *ADA** locus in three different progeny groups derived from a homozygous female are shown in Figure 2.12 and Figure 2.13. Figure 2.12 shows that

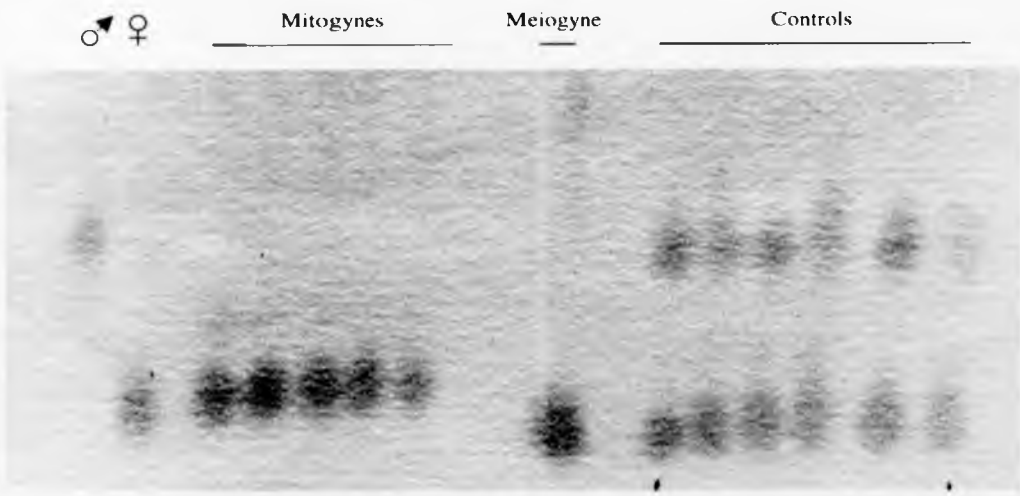


Figure 2.12 Starch gel showing the *ADA** banding pattern of meiotic gynogenetic, mitotic gynogenetic and diploid control progeny groups derived from a homozygous female of *O. niloticus*

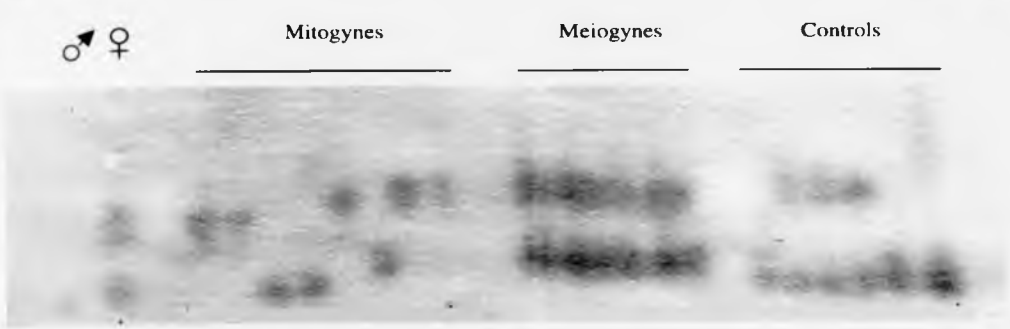


Figure 2.13 Starch gel showing the ADA* banding patterns of meiotic gynogenetic, mitotic gynogenetic and diploid control progeny groups produced from a heterozygous female of *O. niloticus*

in this case both parents were homozygous for *ADA** locus but for different alleles. All meiotic and mitotic gynogenetics have the same alleles as their homozygous mother. On the other hand, all control siblings have two alleles and are heterozygous. Figure 2.13 shows the maternal heterozygosity and paternal homozygosity for *ADA** locus. The mitogynes were homozygous for one or other of the maternal alleles, while the meiogynes have both maternal alleles. All control offspring have one common allele from their homozygous father but the other allele came from either of the two maternal alleles and thus make them homozygous or heterozygous. No paternal alleles were found in any of the gynogenetic groups, suggesting the UV treatment of milt was successful.

In the case of androgenesis all diploid androgens showed blond colouration as their blond father which primarily confirmed the success of the androgenetic techniques used. The karyological analysis of haploid and diploid androgens showed a single set and double set of chromosomes respectively as gynogens in Figure 2.10 and Figure 2.11.

The multilocus DNA fingerprinting was generated by using Jeffreys 33.15 probe which is composed of short, G-C rich tandem repeat sequences. This probe hybridised to the complementary sequences at many polymorphic loci yielded a complex pattern of DNA profile. Figure 2.14 showed that all meiogynes and mitogynes have only maternal

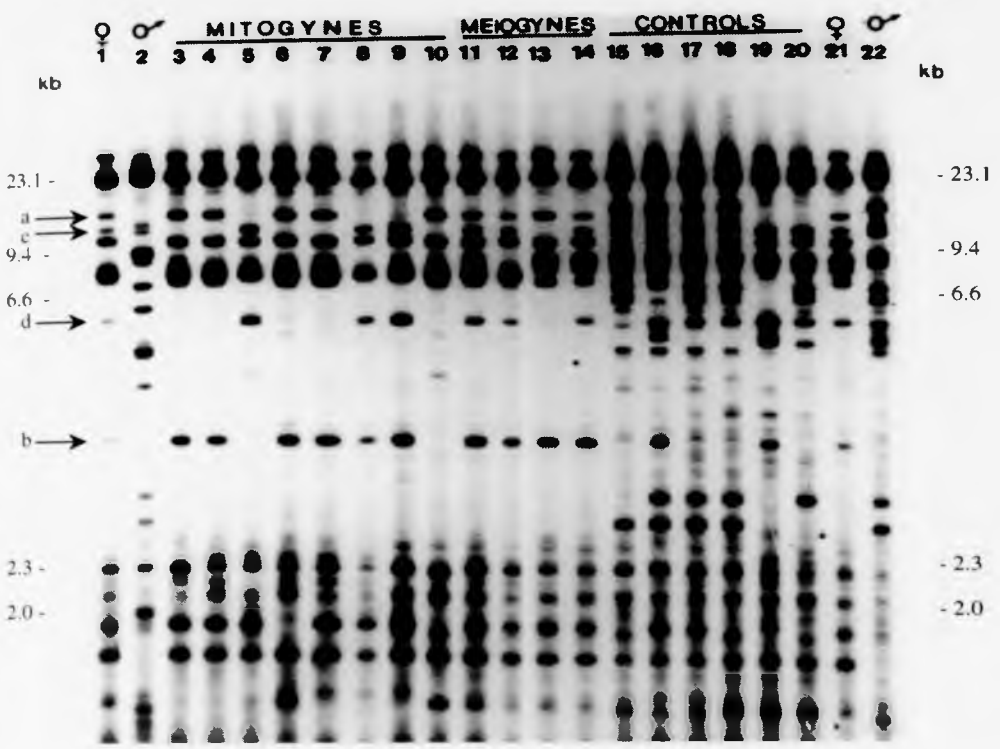


Figure 2.14 DNA fingerprint patterns of tilapia, *O. niloticus*, generated with Jeffreys 33.15 probe.

Keys : Lanes 1 and 21 – mother, lanes 2 and 22 – father, lanes 3-10 – mitotic gynogens, lanes 11-14 – meiotic gynogens, lanes 15-20 – controls. λ –Hind III marker fragments (kb) are positioned at both sides. Bands marked a, b, c and d are referred to in the text.

bands and there were no paternal specific bands in any of the gynogenetic offspring groups. The analysis of segregation of the maternal bands across the meiogynes and mitogynes shows that there are some bands, for example, a and b which are present in all four meiogynes but not in all mitogynes. The segregation of these maternal bands might indicate the homozygosity of the mitogynes for one or other of the maternal alleles. On the other hand, the maternal bands c and d are not present in all the mitogynes as well as in all the meiogynes which shows the dissimilarity in the

segregation pattern of c and d bands with the segregation of a and b. Therefore, it can be suggested that multilocus DNA fingerprinting is useful to evaluate the success of gynogenesis, but it can not be effectively used to distinguish between meiogynes and mitogynes.

The inbred clones produced from mitotic female using subsequent gynogenesis were expected to show no genetic differences between cloned individuals. Figure 2.15

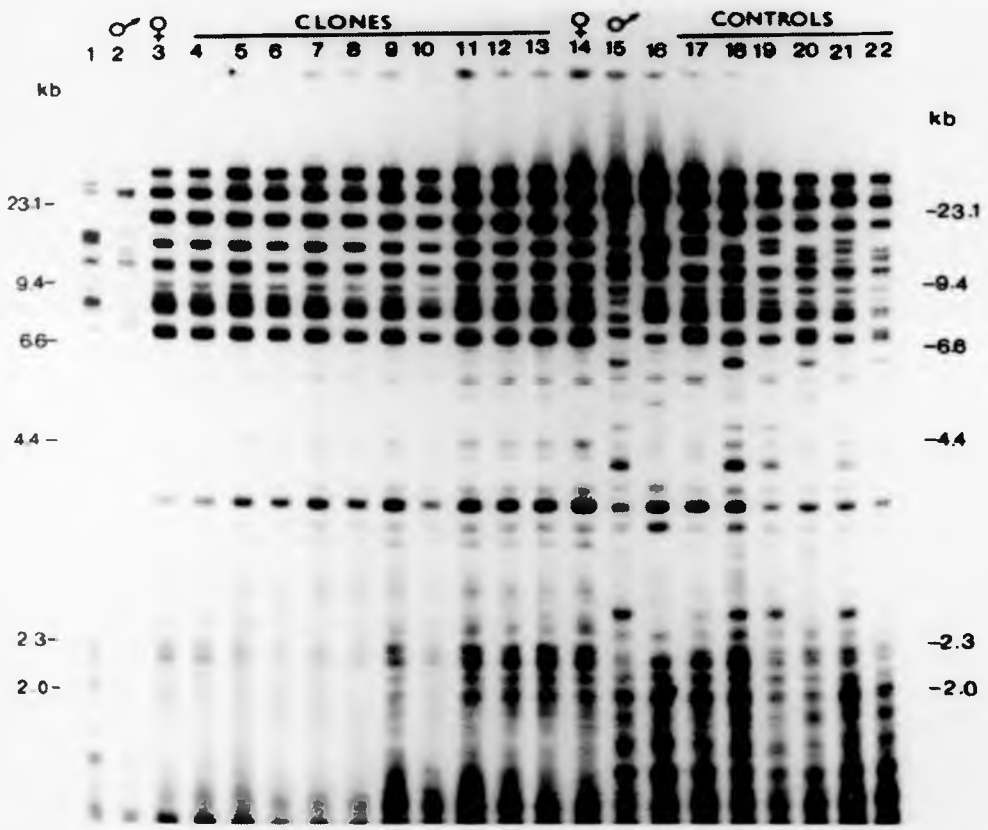


Figure 2.15 DNA fingerprint patterns of gynogenetic cloned tilapia produced from a mitotic gynogenetic mother of *O. niloticus*, generated with Jeffreys 33.15 probe.

Keys : Lanes 1 and 16 – unrelated control, lanes 2 and 15 – father, lanes 3 and 14 – mitotic gynogenetic mother, lanes 4 -13 – clones and lanes 17-22 – controls. Marker fragments (kb) as in Figure 2.14

and Figure 2.16 showed that all of the cloned offspring and their mitotic mother were identical and they were completely free from paternal inheritance. Another set of DNA profiles of inbred clones which were produced from androgenetic inbred female is presented in Fig. 2.17. The inbred clones were produced from the androgenetic female

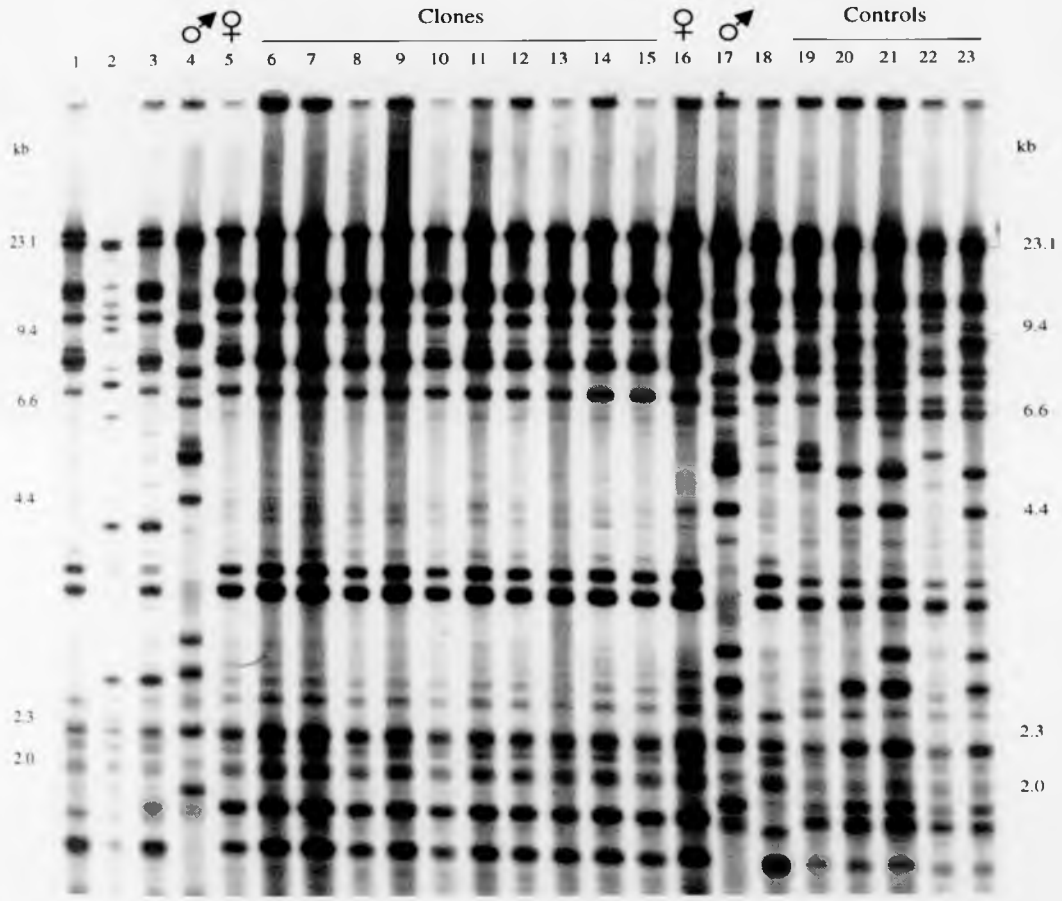


Figure 2.16 DNA fingerprint patterns of gynogenetic cloned tilapia produced from a mitotic mother 002 046 539 of *O. niloticus*, generated with Jeffreys 33.15 probe

Keys : Lanes 1 and 18- unrelated control, lane 2- grand father, lane 3- grand mother, lanes 4 and 17- father, lanes 5 and 16- mother, lanes 6-15- clones, lanes 19-23- controls. Marker fragments (kb) as in Figure 2.14

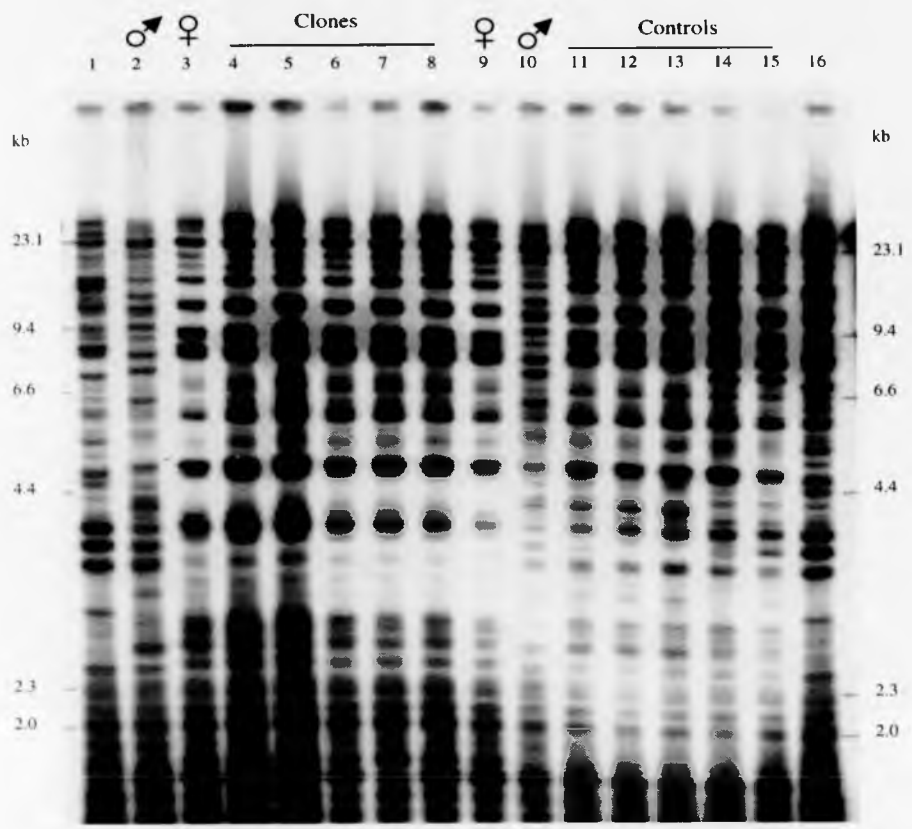


Figure 2.17 DNA fiingerprint patterns of clones produced from an androgenetic inbred female (XX) of *O. niloticus*, generated with Jeffreys 33.15 probe

Keys : Lanes 1 and 16 – unrelated control, lanes 2 and 10 – father, lanes 3 and 9 – mother, lanes 4-8 – clones, lanes 11-15 – controls. Marker fragments (kb) as in Figure 2.14

(XX) by meiotic gynogenesis and were fingerprinted with the 33.15 probe. The fingerprint revealed that all of the bands in clonal individuals are identical to their androgenetic mother and none of the clones have any paternal bands. In the case of heterozygous or outbred clones, the fingerprint showed that all individuals were

identical and shared all bands from both parents as expected by Mendelian inheritance (Figure 2.18). Besides gynogenetic clones, androgenetic clones were also produced from

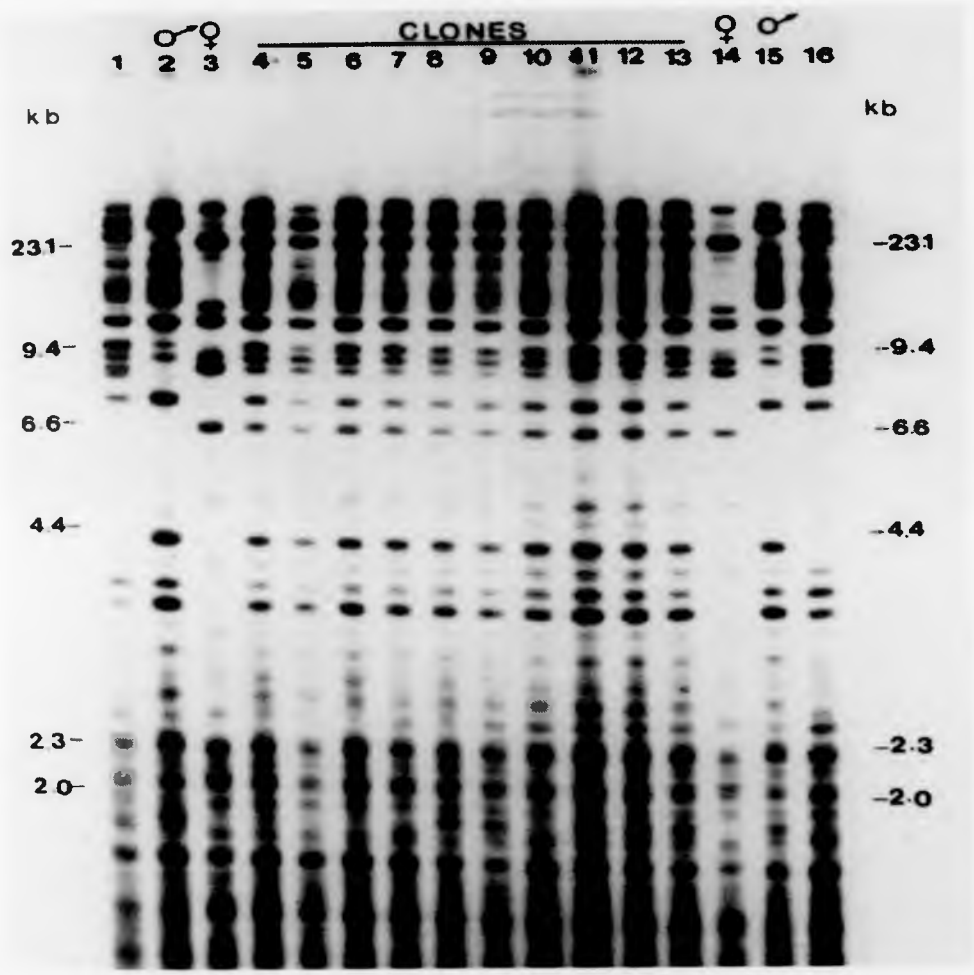


Figure 2.18 DNA fingerprint patterns of gynogenetic heterozygous cloned tilapia, *O. niloticus*, generated with Jeffreys 33.15 probe

Keys : Lanes 1 and 16 – unrelated control, lanes 2 and 15 – father, lanes 3 and 14 – mother, lanes 4-13 – clones. Marker fragments (kb) as in Figure 2.14

androgenetic males by further androgenesis. The fingerprinting of androgenetic clones showed that all the clonal offspring were identical to their androgenetic father and no maternal inheritance was found in such offspring (Figure 2.19).

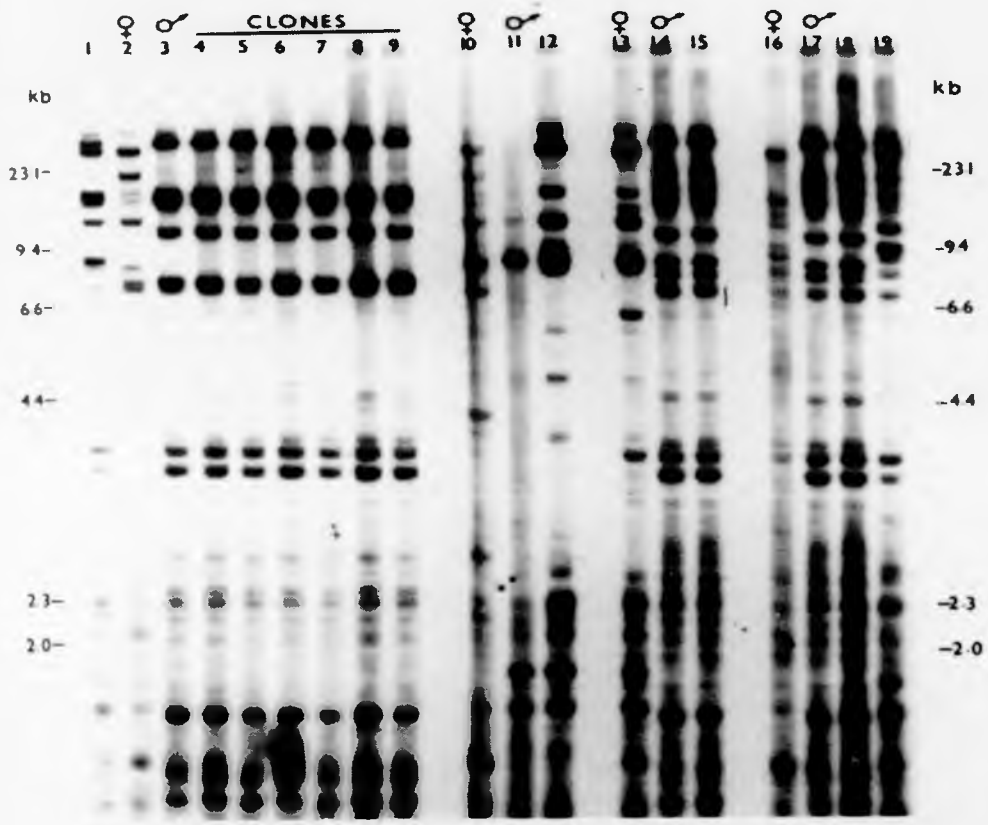


Figure 2.19 DNA fingerprint patterns of different androgenetic cloned tilapia, *O. niloticus*, generated with Jeffreys 33.15 probe.

Keys : Lanes 1 and 19 – unrelated control, lanes 4 -9 – clones produced from mother in lane 2 and androgenetic father in lane 3, lane 12 – clone produced from mother in lane 10 and androgenetic father in lane 11, clone in lane 15 produced from mother in lane 13 and androgenetic father in lane 14, and clone in lane 18 produced from mother in lane 16 and androgenetic father in lane 17. Maker fragments (kb) as in Figure 2.14

2.3.4 Sex ratios of inbred and clonal lines

Sex ratio data obtained from diploid control, meiogyne and mitogyne groups are presented in Table 2.4. Four experiments were carried out and the fishes from all groups were sexed at 12-16 weeks of age. In the second, third and fourth experiments, both meiogynes and mitogynes showed higher number of female progeny, which were significantly different (P<0.05) from 1:1 sex ratio. Sex ratios of controls in the second and third experiments were also significantly different (P<0.05) from 1:1. Sex ratios of meiogynes and mitogynes in the first experiment did not show significant difference and 33.33 - 35.48% males were found in the two groups.

Table 2.4 Sex ratios in diploid controls, meiotic and mitotic gynogenetics of *O. niloticus*. Fish from all the groups were sexed at 12-16 weeks of age using gonad squashing and aceto-carmine staining (Guerrero and Shelton, 1974)

Expt No	Ordinary ♀	Ordinary ♂	Meiogyne			Mitogyne			Diploid control		
			♀	♂	% ♂	♀	♂	% ♂	♀	♂	% ♂
1	010 829 552	000 362 121	20	10	33.33	20	11	35.48	18	14	43.75
2	009 120 547	000 362 121	28	0	0**	25	3	10.71**	12	27	69.23*
3	000 112 323	000 362 121	26	2	7.14**	9	3	25.00**	12	25	67.57*
4	000 367 361	000 362 121	24	2	7.69*	20	2	9.09*	12	15	55.56

* Significantly different from 1:1 sex ratio at P<0.05

** Significantly different from 1:1 and respective control sex ratios at P<0.05

Table 2.5 shows the sex ratio of mitotic gynogenetics in different experiments. Eight separate experiments were conducted. All the experiments produced at least some proportion of males and some of these experiments had surprisingly higher proportion (40-50%) of males. On the other hand, the proportion of females was higher in most

Table 2.5 Sex ratios of mitotic gynogenetics of *O niloticus*. Fish were sexed morphologically without killing as they were used for production of clonal lines. During gynogenesis two males, *O. aureus* (009 037 284) and *O. mossambicus* (009 630 039) were used.

Expt. No	Ordinary ♀	Ordinary ♂	Mitotic gynogenetics		
			♀	♂	% ♂
1	010 829 552	009 037 284	9	2	18.18*
2	000 005 529	009 037 284	2	2	50.00
3	009 120 547	009 037 284	4	1	20.00
4	000 863 847	009 037 284	21	7	25.00*
5	000 770 303	009 037 284	26	4	13.33*
6	000 112 323	009 037 284	12	1	7.69*
7	000 291 557	009 630 039	3	2	40.00
8	000 367 284	009 037 284	4	1	20.00

* Significantly different from 1:1 sex ratio at $P< 0.05$

experiments and a significant excess ($P<0.05$) of female progeny were found in some of these experiments. Mitotic females of these experiments were used to produce clonal lines. The presence of high numbers of males in both meiogyne and mitogyne groups were quite unexpected and the occurrence of such number of males might be the effects of a recessive sex determining gene resulting from inbreeding.

Fish from five clonal lines were sexed at the age of 14 weeks. The result of sex ratios in different experiments was given in Table 2.6. Fish in the first four clonal lines were 100% female but the last clonal line produced a considerable percentage (25%) of male which was significantly different ($P<0.05$) from 1:1 sex ratio.

Table 2.6 Sex ratios of inbred clones produced by meiotic gynogenesis from mitotic gynogenetic females of *O niloticus*. Fish were sexed at the age of 14 weeks.

Expt. No	Mitotic ♀	Ordinary ♂	Clone			Diploid control		
			♀	♂	% ♂	♀	♂	% ♂
1	009 819 347	000 362 121	50	0	0**	16	15	48.39
2	001 354 550	000 362 121	31	0	0**	26	4	13.33**
3	009 356 316	000 362 121	36	0	0**	21	15	41.67
4	010 036 092	000 362 121	42	0	0**	14	11	44.00
5	002 046 539	000 362 121	18	6	25*	15	31	67.39*

* Significantly different from 1:1 sex ratio at P<0.05

** Significantly different from 1:1 sex ratio at P<0.01

2.3.5 Neomales and progeny testing

Neomales were produced from gynogenetic cloned fry by feeding with methyltestosterone treated diet. The neomales were used to produce heterozygous or outbred clones.

The outbred clones were produced by crossing neomales with mitotic females where they represent two different lines. Diploid controls were also produced by crossing between neomales and an ordinary female. Five different neomales were used in outbred clone production as well as ordinary controls. Fish from both groups were sexed and the observed sex ratios were presented in Table 2.7. All fish in both groups were 100% female which were significantly different (P<0.01) from 1:1 sex ratio. The sex results demonstrated absolute success of sex reversal experiments. Here the experiments with ordinary controls can be considered as progeny testing of neomales (XX). One androgenetic cloned male (YY) was crossed with a mitotic and an ordinary female. Both crosses produced 100% male which was significantly different (P<0.05) from 1:1 sex ratio.

Table 2.7 Results of sex ratios in outbred clones. The outbred clones were produced by crossing between neomales (XX) and mitotic females from different inbred lines of *O. niloticus*. The last group of outbred clones was produced by crossing between a mitotic female and an androgenetic cloned male (YY). For control groups each male was separately crossed with an ordinary female. Fish were sexed at the age of 16 weeks

Tag no. of Neomales ♂	Mitotic ♀	Outbred clones			Ordinary ♀	Diploid control		
		♀	♂	% ♂		♀	♂	% ♂
006 338 797 (XX)	009 823 379	56	0	0**	011 020 109	36	0	0**
009 353 844 (XX)	010 036 092	65	0	0**	011 020 109	41	0	0**
009 783 894 (XX)	009 356 316	43	0	0**	011 020 109	39	0	0**
014 526 044 (XX)	009 356 316	47	0	0**	011 020 109	37	0	0 **
000 292 305 (YY)	009 356 316	0	52	100**	011 020 109	0	40	100**

** Significantly different from 1:1 sex ratio at P<0.01

Table 2.8 shows progeny testing results of neomales (XX) originating from a mitotic mother 002 046 539 which produced some proportion of males in her clonal line. All the neomales produced around 50% male progeny through crossing with an apparently ordinary female 11C and the sex ratios were not significantly different from 1:1 ratio. These neomales also produced 100% female when they were crossed with an another female 005 276 046. However, any maternal effects on male production were checked by backcrossing the neomales with their mother. The backcrosses produced more than 50% males and the sex ratios were not significantly different from 1:1 except one group. The crosses of these neomales with both female 11C and the mitotic mother produced high proportions of males, but statistical analysis showed that the latter crosses had a significantly higher (P<0.05) proportion of males than the crosses with female 11C. Although the neomales produced both sexes in the above two crosses they

Table 2.8 Results of sex ratios in crosses of neomales (XX) of *O. niloticus* with two founder females and their mitotic mother (002 046 539). They were gynogenetically produced from the mitotic female and sex resversed by hormone treatment using 17 α–methyl testosterone. Fish in all the groups were sexed at 16 weeks of age.

Neomales (XX) ♂	Founder ♀	Sex			Founder ♀	Sex			Mitotic mother	Sex		
		♀	♂	% ♂		♀	♂	% ♂		♀	♂	% ♂
006 271 336	11 C	17	15	46.88	005 276 046	35	0	0**	002 046 539	9	17	65.38
006 582 276	11 C	9	8	47.06	005 276 046	38	0	0**	002 046 539	6	11	64.71
006 517 057	11 C	16	16	50.00	005 276 046	36	0	0**	002 046 539	9	11	55.00
006 887 008	11 C	23	14	37.84	005 276 046	26	0	0**	002 046 539	6	16	72.73*
Mean				**45.45 ± 2.63**				**0**				**62.27 ± 2.44**
006 554 347#	11 C	19	13	40.63	005 276 046	41	0	0**		-	-	-
000 362 121 (normal male)	11 C	25	19	43.18	005 276 046	17	10	37.04	002 046 539	21	48	69.57*

* Significantly different from 1:1 sex ratio at $P < 0.05$

** Significantly different from 1:1 sex ratio at $P < 0.01$

Neomale died before it could be backcrossed to dam

are definitely clones. They also produced 100% females in the crosses with female 005 276 046. The fingerprints of meiotic progenies (including these neomales) of the mitotic female 002 046 539 confirmed their clonal nature (Figure 2.16). All meiotic offspring were identical to their mitotic mother and none of them had any paternal inheritance. Therefore the female 11C which produced both male and female progenies is assumed not to be an ordinary XX female. It might be an XY female or the production of males by this female might be the effects of autosomal sex modifying loci or some other genetic factors, as appears to be the case within the clonal line derived from female 002 046 539.

Seven androgenetic cloned males (YY) were progeny tested by crossing them separately with an ordinary female and all of the resultant progenies showed 100% male sex (Table 2.9). The sex ratios were significantly different ($P<0.01$) when tested against a theoretical 1:1 sex ratio.

Table 2.9 Progeny- testing of androgenetic cloned males (YY) produced from androgenetic Inbred males of *O. niloticus*. Fish were sexed by gonad squashing and aceto-carmine staining (Guerrero and Shelton, 1974) at 3-4 months of age

Tag no. (YY) ♂	Ordinary ♀	No. of progeny tested	Sex		
			♂	♀	% ♂
000 367 321	011 020 109	42	42	0	100**
000 032 628	011 020 109	81	81	0	100 **
009 380 044	011 020 109	100	100	0	100**
009 527 066	011 020 109	43	42	1	97.67**
009 595 335	011 020 109	66	66	0	100**
000 292 305	011 020 109	30	30	0	100**
000 119 833	011 020 109	32	32	0	100**
000 362 121 (Control)	011 020 109	54	22	32	40.74

** Significantly different from 1:1 sex ratio at $P<0.01$

2.4 Discussion

Since the present study mainly focused on the production of inbred lines and their clones and their use in immune response experiments, more emphasis was given to their production rather than standardisation of UV irradiation, intensity and timing of heat shock treatments. Gynogenesis and androgenesis were carried out by following the procedures described by Hussain *et al.* (1993) and Myers *et al.* (1995) respectively. In this study, UV dose and its treatment time were effective in the production of gynogenetics and androgenetics, and their clones. In gynogenesis, the UV dose of 250-265 $\mu W\ cm^{-2}$ for 2 mins caused complete breakdown of sperm's DNA, although the UV dose was lower than 300-310 $\mu W\ cm^{-2}$ used for tilapia sperm irradiation (Hussain *et al.*, 1993). Although both ranges of UV doses were effective for complete inactivation of sperms DNA, the lower dose yielded comparatively higher percentage of mitogynes (6.88%) than the higher dose (2%). The successful inactivation of sperm using UV light of 250-265 $\mu W\ cm^{-2}$ indicates that *O. niloticus* spermatozoa can be inactivated by a lower UV dose than the above reported dose of 300-310 $\mu W\ cm^{-2}$. In fact, the UV dose used in this study was not standardised through a series of dose trials. Primarily, the higher UV dose (300-310 $\mu W\ cm^{-2}$) was used to inactivate sperm's DNA but it produced less fertilisation of eggs which might be resulted from the inability of fertilisation of severely damaged sperms caused by higher UV dose. The UV dose was reduced from 300-310 $\mu W\ cm^{-2}$ to 250-265 $\mu W\ cm^{-2}$ based on discussion with Dr Jim Myers (Who was working on gynogenesis and androgenesis of tilapia in the same Reproduction and Genetics laboratories).

In androgenesis, UV treatment was also effective for complete disruption of the egg nuclear DNA. Disruption of egg DNA was carried out by a UV treatment of 4 mins at 150 $\mu W\ cm^{-2}$, although Myers *et al.* (1995) mentioned that an administration of UV treatments from at least 5 mins to up to 10 mins at 150 $\mu W\ cm^{-2}$, total dose 450-900 $J\ m^{-2}$, was necessary for complete oocyte denucleation. Bongers *et al.* (1994) were able to produce higher numbers of androgenetic haploids (53.9%), relative to control, in common carp, *C. carpio* using an optimum UV dose of 2500 $J\ m^{-2}$. Masaoka *et al.*

(1995) produced androgenetic diploids in loach (*Misgurnus anguillicaudatus*) by inactivating the egg genome using UV irradiation with a dose of 750 J m^{-2}.

Heat treatments applied in the production of diploid gynogenetics, androgenetics and their clones were successful. For mitotic gynogenesis, first cleavage was suppressed at 27-29 mins post fertilisation using 42.0 to 42.5 °C heat shock for 4 mins which was similar to 42.5 °C for 3 or 4 mins applied at 22.5–30 mins post fertilisation (Myers *et al.*, 1995) but higher than that reported for mitotic inhibitions in tilapia by Hussain *et al.* (1993) at 41.0 ± 1 °C for 3.5 mins at 27.5 mins post fertilisation. The heat shock times for the production of mitogynes were similar in all three studies, but the present study gave an average survival of 6.88%, to yolk-sac resorption relative to control, where the two other previous yields were 2.0% (Hussain *et al.*, 1993) and 10.6% (Myers *et al.*, 1995) respectively. In *O. aureus*, the production of mitotic gynogens was not satisfactory and the induction of mitotic gynogenesis resulted in lower survival rate with a mean of 0.8% to yolk-sac resorption (Mair, 1988). The observed survival rate of mitogynes in this study was lower than the previous result (10.6%) in the same fish (Myers *et al.*, 1995) and much lower than other reported fish, e.g. 20% in zebra fish (Streisinger *et al.*, 1981), 15.7% in common carp (Komen *et al.*, 1991) and 16% in rainbow trout (Quillet *et al.*, 1991). The low survival of mitotic gynogenetic fry in different fish species might be due to higher rate of expression of recessive deleterious and lethal genes in homozygous fry which might cause their death (Onozato, 1984; Lou and Purdom, 1984b; Purdom *et al.*, 1985; Mair *et al.*, 1987) or different sensitivities to the treatments used.

In the analysis of sex frequencies in gynogenetic offspring of *O. niloticus*, one of the families showed a high percentage of males in both meiotic and mitotic gynogenetics. Fish from other families produced almost 100% female sex in meiogynes and mostly females in mitogynes. Hussain *et al.* (1994a) obtained 7.5% and 47.5% males in the meiotic and mitotic gynogenetic progenies of *O. niloticus* respectively and suggested that an epistatic locus (SDL-2, two alleles, *SR* and *sr*) causing sex reversal from female to male under homozygous condition. Thus a female which is heterozygous

at this locus (XX *SRsr*) would be expected to produce non-recombinant homozygous males (XX *srsr*) and both non-recombinant and recombinant females (XX *SRSR* and XX *SRsr* respectively) in meiotic gynogenesis and only XX *srsr* males and XX *SRSR* females in mitotic gynogenesis. Mair *et al.* (1991a) observed 4.1% males in meiogynes and 20% males in homozygous mitogynes of *O. niloticus*. They suggested that the occurrence of males in gynogens may result from some form of "natural sex reversal" of females and this sex reversal mechanism develops from homozygosity of rare autosomal, recessive, sex-influencing genes. About 50% male progeny in the mitogynes of *O. niloticus* was reported by Andreas and Gabriele (1995). Komen (1990) observed that the frequency of mitotic males was consistent with the ratio of 1 : 1.34 to male : female in the mitotic population of common carp.

According to Jalabert *et al.* (1974) and Hopkins (1979), males in *O. niloticus* are heterogametic (XY) and females are homogametic (XX), so, in gynogenesis both meiotic and mitotic gynogens should be exclusively female (Penman *et al.*, 1987a). In the production of mitotic gynogenetics from *O. niloticus*, Mair (1988) observed that a certain proportion of mitogynes were male. He thought that the occurrence of mitotic male might be an output of a spontaneous sex reversal event controlled by a combination of rare autosomal sex modifying loci. In the occurrence of the male sex in the mitotic gynogenetics of common carp, it was assumed that the mother of these males was heterozygous for a recessive mutation in a minor sex determining gene which in the homozygous condition induces a testis or intersex gonad in XX offspring (Komen and Richter, 1993).

In the present study, inbred or homozygous clones of *O. niloticus* were produced in two ways. Firstly, clones were produced from mitotic gynogenetic females using further gynogenesis, and secondly, by crossing between neomales and their mitotic mother. A big difference in mean survival rate at yolk-sac resorption stage was found between the two clone production methods used, 9.62% in the first method and 43.23% in the later. The comparatively lower survival of the inbred clone production by the first method might be due to the sensitivity of the eggs of homozygous mitotic females to

heat shock treatment (Hussain, 1992). The mean survival of the outbred or heterozygous clones at both pigmentation and yolk-sac resorption stages were 45.17% and 35.40% respectively. The mean survival rate of homozygous clones at yolk-sac resorption stage was apparently higher than that of heterozygous clones but not significantly different. Although the survival rate of the heterozygous clones were less than the homozygous clones, no abnormal or deformed fry were found in this clone group. In contrast, some abnormal or deformed fry were nearly always observed in inbred clonal groups, especially in the clones produced by the gynogenesis method. The heterozygous clones were generally looked more active and their growth performances were better than the inbred clones. The superiority of the outbred clones over the inbred clones might be the expression of heterosis resulting from the cross between two different homozygous lines as has been reported by Cherfas (1981) and Nagy (1987). Streisinger *et al.* (1981) observed better performance of hybrid clones in zebrafish, *B. rerio*, which were produced by crossing between homozygous individuals.

In the present study, both inbred and outbred clonal lines exhibited 100% female sex except one inbred line which showed a considerable percentage (25%) of male progeny. It is not easy to give a definite explanation of the above mentioned phenomenon but there are some possibilities :

i) if the mother (002 046 539) of this clonal line is not a mitotic gynogenetic homozygous female, she might be an XY female. Meiogynes from this female would be mostly XY males and these males would be unlikely to give all female progeny in any progeny testing. However, the neomales from this clonal line produced 100% female progeny through crossing with a female 005 276 046. Another mitotic female 002 041 887 (she was the sister of the female 002 046 539) also produced males in her clonal line. Neomales produced from this line when crossed with an ordinary female produced all female offspring. So, the findings of the sibling clones supported that both 002 041 887 and 002 046 539 fish were mitotic females.

ii) the mitotic mother might have got paternal chromosomal fragments. The sex determining mechanism should be influenced by this partial paternal inheritance, but nothing showed up in fingerprints.

iii) other genetic factors such as natural sex reversal (Mair *et al.*, 1991a) or environmental factors might controlling the sex differentiation mechanisms. Streisinger *et al.* (1981) reported the occurrence of homozygous gynogenetic cloned males in zebrafish and suggested that the variation in sex ratio was not only dependent on female's homo or heterogametic system but possible autosomal sex determining genes or environmental effects, like high temperature (Abucay *et al.*, 1997).

The cross between the neomales and their mitotic mother is probably between identical, completely homozygous individuals. In other similar crosses used to produce clonal lines, only females were obtained. If this clonal line was fixed for something genetic with a strong effect (e.g. some "strength" as the XX/XY system) it might expect that all of the progeny would be the same sex (male?). However, as this phenomenon is not occurred here (also the mother and most of the other fish in the first batch of clones produced by meiotic gynogenesis were female), it seems likely that the clonal line is fixed genetically at some allele or combination of alleles at different loci which cause greater susceptibility to environmental effects (≡ greater instability in sex differentiation).

The results of progeny testing of other clonal neomales (XX) revealed their absolute performance of 100% female sex production (Table 2.7). The sex ratio results also indicated the successful reversal of females to male by the hormone treatment at the early fry stage. These neomales are potentially very valuable for aquaculture research, especially for monosex production. By crossing the neomales with any female (XX), the resultant progeny will be all female. Another alternative way to produce monosex fry is by using androgenetic cloned males (YY). In this study, comparatively little effort was

paid to producing androgenetic clones. So far few androgenetic clones have been produced and the progeny testing (Table 2.9) of these clones revealed them to be completely homozygous for the sex determining locus.

The nature of the gynogenetic inbred fish and their clones were verified by using the isozyme locus *ADA**. The *ADA** banding patterns generated from three groups of progeny in a single family (Figure 2.13) showed that all meiogynes have same alleles as their heterozygous mother and indicated their heterozygosity resulted from recombination of genes distal to the centromere. The mitogynes were homozygous for one or other of the maternal alleles. No paternal inheritance was found in any individuals of either type of gynogenetic groups. In contrast, the controls were all heterozygous with alleles from both of the parents. Similar patterns of band segregation in meiotic and mitotic gyneogenetics and controls were also reported by Mair (1988) and Husssain *et al.* (1993).

DNA fingerprints presented in this study revealed their potential to interpret parental inheritance in both gynogenetic and androgenetic offspring. It has been shown that the hypervariable 33.15 DNA probe (Jefferys *et al.*, 1985) can be used to produce individual-specific DNA fingerprints from tilapia, *O. niloticus*. The analyses of the fingerprints showed that individual parental-specific bands are obvious in their subsequent offspring depending on the nature of their production, but the DNA fragments over the whole gel should be categorised into two groups. The fragments of 4.0 k bp and above could easily be detected, but most of the fragments of 4.0 k bp and below were very poorly resolved and close to each other, thus making them indistinguishable. The clustering of the small fragments in the lower portion of the gel may be due to the use of a lower agarose gel concentration (0.7%). Clearer resolution of the small fragments was possible if higher agarose concentrations (e.g. 2%) were used (Figure 2.8). In fact higher agarose concentrations can produce somewhat more clear resolution of the small fragments but it is not clear enough to detect individual bands. In addition to this gels with higher agarose concentration were comparatively harder, which may make difficulties during transferring DNA to a membrane.

Georges *et al.* (1988) reported the potential of DNA fingerprinting for studying gynogenesis in fish. They used four DNA probes to detect individual-specific fingerprints in the barbel, *Barbus barbus* and recognised that different probes produce different complex fingerprints in the same animal. In the present study, the analysis of the fingerprints of gynogenetic offspring along with related diploid controls revealed that all of the bands in meiogynes came from mother, but their banding pattern was not similar to their mother. This is because some of the maternal bands were segregated in the meiogynes. The mitogynes shared bands to their mother but individual-specific for their homozygosity for their respective maternal alleles. A number of DNA probes are now available and each of them has potential to produce individual-specific DNA profiles. Carter *et al.* (1991) stated that human minisatellite probes (pSPT 18.15 and pSPT 19.6) can be used successfully to produce fingerprints for analysing gynogenesis in tilapia. It can detect the maternal inheritance in the gynogenetic offspring but without assigning individual bands to specific loci, successful mitotic gynogenesis can not be proved. Harris *et al.* (1991) observed that the most commonly used DNA probes 33.15 and 33.6 were able to generate individual-specific DNA fingerprints in *O. niloticus*, but more information can be obtained from fingerprints produced by the 33.6 than by the 33.15 probe. Gross *et al.* (1994) produced nest-specific DNA fingerprints in smallmouth bass, *Micropterus dolomieu*, by using the 33.15 DNA probe and successfully identified smallmouth bass individuals originating from a specific nest in a mixed population of bass in Lake Ontario.

Another chemically synthesised probe "M13" has also been used and reported in different fish. It has been used to produce fingerprints in rainbow trout, *Onchorynchus mykiss* (Fields *et al.*, 1989), in barbel, *Barbus barbus* (Georges *et al.*, 1988) and in ayu, *Plecoglossus altivelis* (Han *et al.*, 1992). Vassart *et al.* (1987) have shown that a specific DNA sequence in bacteriophage M13 reveals polymorphic patterns of restriction fragments in human and other animals that are different from the patterns reported by Jeffreys *et al.* (1985).

DNA fingerprinting has great potential for aquaculture research. As it can produce individual-specific patterns, fingerprinting can be used for identification of individuals, construction of pedigrees and population genetic studies (Hallerman and Beckman, 1988). In addition to producing individual-specific patterns, researchers tried to exploit this technique to generate family-specific fingerprints (Gilbert *et al.*, 1990; Wirgin, *et al.*, 1991) which can be used to monitoring families position in a common population where tagging or any individual identification is not feasible. The use of DNA fingerprinting in the present study confirmed the specific nature of gynogenetics, androgenetics and their clones.

Chapter III

Major histocompatibility complex genes and evidence for their occurrence in the tilapia, *Oreochromis niloticus*

3. Major histocompatibility complex genes and evidence for their occurrence in tilapia, *Oreochromis niloticus*

3.1 Introduction

3.1.1 Major histocompatibility complex and its functional organization in mammals

Major histocompatibility complex (MHC) is recognised as an important set of genes responsible for controlling whether grafts are accepted between individuals whose tissues are genetically similar (histocompatible) or rejected by individuals whose are not (histoincompatible). The MHC contains a set of genes located together on one chromosome as a complex. They encode several series of families of polymorphic glycoproteins, including two families of molecules that are expressed at the cell surface, the class I and class II molecules. These specialised membrane proteins act as a guidance system that allow T cells to recognise antigen.

3.1.1.1 Structure of class I and class II molecules

Each class I molecule consists of a heavy α chain, which is noncovalently associated with a light chain, the β_2 microglobulin (Grey *et al.*, 1973; Nakamuro *et al.*, 1973). The α chain is a polymorphic transmembrane glycoprotein of about 45 kilodaltons (KD) encoded by class I MHC loci, whereas the β_2-microglobulin is an invariant protein of about 12 KD encoded by another gene located on a separate chromosome (reviewed in Ploegh *et al.*, 1981). Although β_2-microglobulin is not located in the MHC, its association with class I molecules is required for the expression of MHC on cell membranes (Goodfellow *et al.*, 1975; Ploegh *et al.*, 1979 and Rein *et*

al., 1987). Recent experiments suggest that the binding of a peptide to a class I MHC α chain may induce a conformational change in the molecule, enabling it to associate with β_2-microglobulin and then be transported to the cell membrane.

The α chain of class I MHC molecules is organized into three external domains (α_1, α_2 and α_3), each having approximately 90 amino acids, a transmembrane domain of about 40 amino acids and a cytoplasmic anchor segment of 30 amino acids. It is anchored in the plasma membrane by its hydrophobic transmembrane segment and hydrophilic cytoplasmic tail. According to size and organization, β_2-microglobulin is similar to the external domains of the α chain, and its sequence analysis shows that there is a considerable homology between the α_3 domain , β_2- microglobulin and the constant-region domains of immunoglobulins. Class I MHC molecules and β_2 - microglobulin are therefore, classified as members of a immunoglobulin superfamily (Kuby, 1997; Williams and Barclay, 1988). The α_3 domain appears to be highly conserved among class I MHC molecules and contains a sequence that is recognised by the CD8 (cluster determinant) T cell membrane molecule. The β_2-microglobulin interacts extensively with the α_3 domain and also interacts with amino acids of the α_1 and α_2 domains. The interaction of β_2-microglobulin appears to be necessary for the proper conformation of the class I MHC molecules (Kuby, 1997).

The structure of class II MHC molecules is similar to that of class I molecules in that they are membrane-bound glycoproteins containing external domains, a transmembrane segment, and a cytoplasmic anchor segment. Each chain in a class II molecule contains two external domains : α_1 and α_2 domains, and β_1 and β_2 domains. The membrane proximal α_2 and β_2 domain, like the membrane-proximal α_3 domain of

class I MHC molecules, possess sequence homology to the immunoglobulin-fold domain structure and for that reason, class II MHC molecules are also classified in the immunoglobulin superfamily.

The chromosomal loci that encode class I and class II MHC molecules are the most polymorphic known in higher vertebrates, i.e. within a given species, there are extraordinarily large number of different alleles at each locus (Klein, 1986; Rothbard and Gefter, 1991). In mice, more than 55 alleles have been identified at the K locus and 60 alleles at the D locus. A comparison of the amino acid sequences of several allelic MHC molecules encoded at a single locus reveals a sequence divergence of between 5 and 10%. This degree of variation is unusually high. The sequence variation among MHC molecules is not randomly distributed along the entire polypeptide chain, but instead is clustered in short stretches, largely within the α_1 and α_2 domains of class I molecules and within the α_1 and β_1 domains of class II molecules. A number of researchers have suggested that the differences in these polymorphic amino acids in the class II MHC molecules expressed on antigen-presenting cells, might influence the cells' ability to recognise a given peptide (Kuby, 1997).

3.1.2 Cells involved in the immune system

3.1.2.1 B-lymphocytes

B-lymphocytes express a unique antigen-binding membrane receptor which is called the antibody molecule. The antibody molecule is composed of two identical heavy polypeptide chains and two identical light polypeptide chains held together by disulfide bonds. The amino-terminal ends of each heavy and light-chain (V_H and V_L) constitute a variable sequence and form a cleft within which antigen binds. When a B

cell encounters the antigen for which its membrane-bound antibody is specific, the cell begins to divide rapidly, its progeny differentiate into memory B cells and plasma cells that secrete soluble antibody proteins. Memory B cells have a longer lifespan and continue to express membrane-bound antibody with the same specificity as the original parent cell. Plasma cells do not express membrane-bound antibody, but secrete enormous amounts of antibody during their short life period.

3.1.2.2 T-lymphocytes

T-lymphocytes express a unique membrane receptor for antigen. The receptor is a heterodimer, composed of two protein chains, either $\alpha\beta$ or $\gamma\delta$, which are linked by disulfide bonds. The amino-terminal ends of the chains fold together to form the antigen-binding cleft of the T cell receptor.

T cells are of two types :

i) T helper cells (T_H cells) which are restricted for MHC class II molecules and display CD4 membrane glycoproteins.

ii) T cytotoxic cells (T_C cells) which are restricted for MHC class I molecules and display CD8 membrane glycoproteins.

The CD4 and CD8 glycoprotein molecules are invariant in structure and are thought to be accessory molecules which play a role in the interaction of T cells with non-T cells. These glycoproteins appear on the surface of the T-cells just before the appearance of the T-cell receptor during their maturation in the thymus. CD4 acts as a marker of T_H cell populations which promote activation and maturation of B-cells and cytotoxic T-cells, and control antigen-specific chronic inflammatory reactions through stimulation of macrophages. These molecules form links with class II MHC on the cell presenting antigen. Similarly, the CD8 molecules on the surface of cytotoxic T-cells

associate with MHC class I molecules. An important difference between CD4 and CD8 cells is that CD4 cells are activated by signals provided both by an antigen-MHC class II molecule complex and IL-1 (Interleukin) provided by the antigen presenting cells. On the other hand, the proliferation and differentiation of CD8 cells depend on a signal provided by the antigen-MHC class I complex and an additional signal, IL-2 which is released as a consequence of the activation of CD4 helper T-cells.

In response to the recognition of antigen by the receptor in conjunction with the MHC molecule, a T_H cell secretes various growth factors, which are collectively known as lymphokines. When a T_H cell is activated, it becomes an effector cell secreting various lymphokines which play an important role in activating B cells, T_C cells, phagocytic cells, and various other cells that participate in the immune response. Under the influence of T_H-derived lymphokines, when the T_C cell receptor together with MHC molecules recognises an antigen, it proliferates and differentiates into effector cells called Cytotoxic T Lymphocyte (CTL). In contrast to the T_H cell, the CTL does not generally secrete lymphokines and instead acquires cytotoxic activity. The CTLs monitor the cells of the body and eliminate any that display antigen, such as virus-infected cells, tumor cells, and cells of a foreign tissue graft. Such cells displaying foreign antigen complexed to an MHC molecule are called altered self-cells.

3.1.2.3 Antigen presenting cells

The activation of both the humoral and cell-mediated branches of the immune system depends on the production of lymphokines from T_H cells. The T_H cells can be activated following antigen recognition only when the antigen is displayed together with MHC on the surface of specialized cells called antigen-presenting cells (APCs). The antigen presenting cells include macrophages, B cells, and dendritic cells; these are

distinguished by their expression of a particular type of MHC molecule. These specialized cells internalize antigen, either by phagocytosis or by endocytosis, and then re-express a part of that antigen, together with the MHC molecules, on their membrane. The T_H cell then recognises the antigen associated with the MHC molecule on the membrane of the antigen-presenting cell.

Antigens which are generally very large and complex, are not recognised in their entirety by T or B lymphocytes. Instead both T and B lymphocytes recognise discrete sites on the antigen called antigenic determinants, or epitope. Epitopes are the immunologically active regions on a complex antigen, the regions that actually bind to B or T cell's receptor. A comparison of the amino acid sequences of the V_L and V_H domains of B cell receptor reveal that the amino acid sequence variability is concentrated in several hypervariable regions. These regions form the antigen binding site of the antibody molecule. Because the antigen-binding site is complementary to the structure of the epitope, the hypervariable regions are also called complementary-determining regions (CDRs).

The most important difference in antigen recognition by T lymphocytes and B lymphocytes is that B cells can recognise an epitope alone, whereas T cells can only recognise an epitope when it is present on the surface of a self-cell in association with an MHC molecule. For a T cell to recognise a foreign protein antigen, it must be degraded into small peptides that form physical complexes with a class I or class II MHC molecule. This conversion of proteins into MHC-associated peptide fragments is called antigen processing. Figure 3.1 presents the schematic diagram of the cellular immune system (cited from Kuby, 1997).

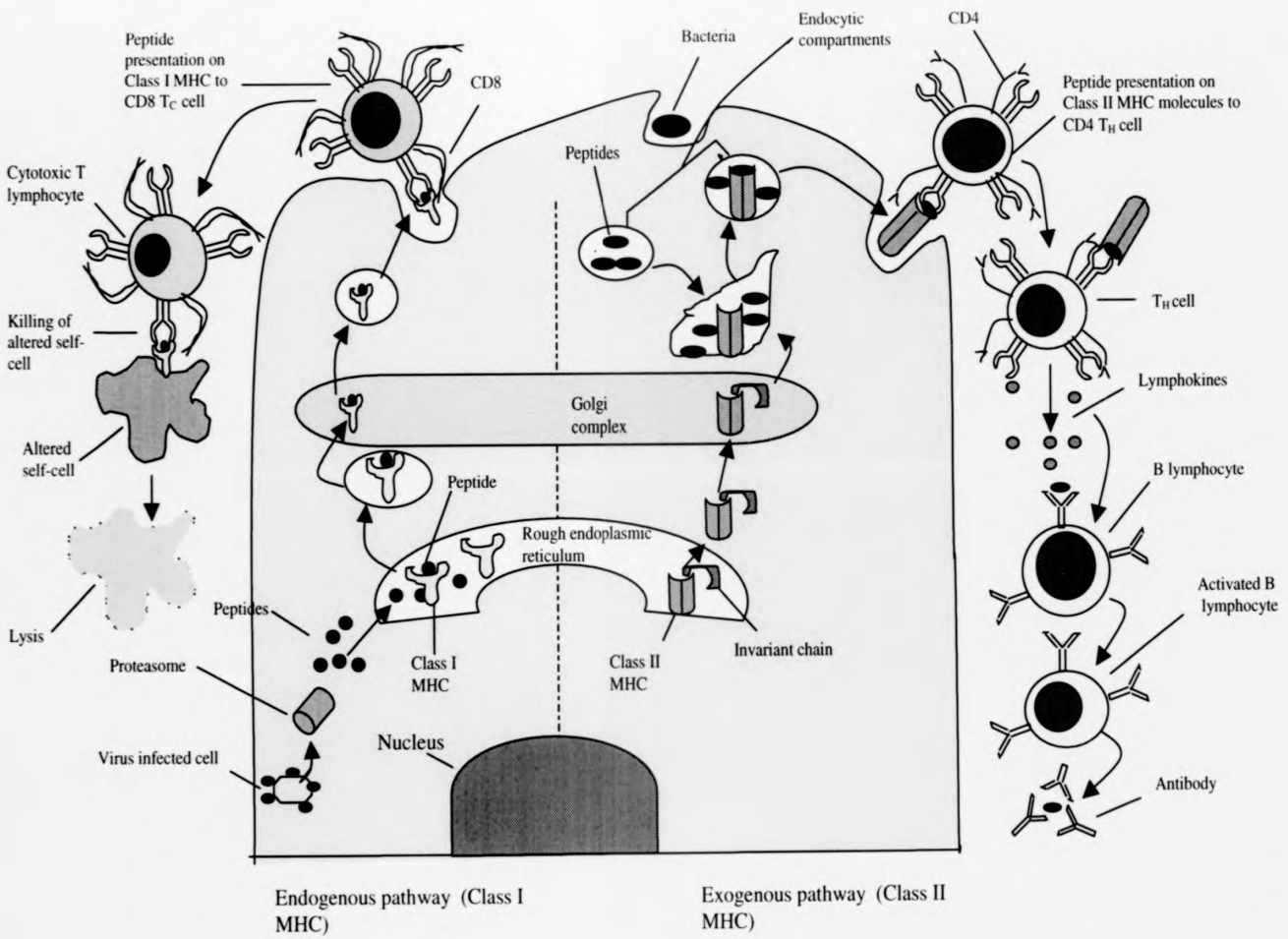


Figure 3.1 Schematic diagram of the antigen presenting cells involved in cellular immune system (Cited from Kuby, 1997)

Exogenous antigens (e.g. bacteria, parasites) are internalized by phagocytosis or endocytosis of antigen-presenting cells. Macrophages can internalize antigen by both processes, whereas most other APCs are not phagocytic or are poorly phagocytic and therefore internalize exogenous antigen only by endocytosis (either receptor-mediated endocytosis or pinocytosis). After internalized an antigen, it is degraded into peptide fragments within compartments of the endocytic processing pathway where the class II MHC molecules are expressed. The endocytic processing pathway appears to involve three increasingly acidic compartments : early endosomes (pH 6.0-6.5); late endosomes or endolysosomes (pH 5.0-6.0); and lysosomes (pH 4.5-5.0). The internalized antigen moves from early to late endosomes and finally to lysosomes, encountering hydrolytic enzymes and an increasingly low pH in each compartment. The antigen is degraded into oligopeptides in the compartments of the endocytic pathway and bind to the class II MHC molecules. The MHC class II molecule bearing the peptide is then exported to the cell surface where it is recognised by $CD4^+$ T_H cells (Lanzavecchia, 1990). $CD4^+$ binds to the β_2 domains of class II MHC molecules. After recognising and forming a complex with an antigen-MHC class II molecules, the T_H cell becomes activated, begins to divide and give rise to a clone of effector cells. Each of the effector cells is specific for the same antigen-class II MHC complex. These effector cells secrete lymphokines which activate B cells. The B cells then divide and differentiate into plasma cells that secrete antibody proteins. The soluble antibody molecules bind to the antigen and neutralize them or precipitate their destruction by complement enzyme.

Endogenous antigen is produced within the host cell itself. Viral proteins are synthesised within virus-infected host cells. Endogenous antigens are thought to be degraded into peptide fragments that bind to class I MHC molecules within the endoplasmic reticulum. The peptide-MHC complex is then transported to the cell

membrane and recognised by $CD8^+$ T_C cells (Bjorkman and Parham, 1990). Like $CD4^+$, $CD8^+$ binds to the α_3 domain of MHC class I molecule. After forming a complex with antigen-class I MHC molecule, T_C cell proliferates and differentiates into CTL. The CTLs are activated by interaction with an antigen-MHC class I complex on the surface of an altered self-cell and kill it. In the killing process the $CD8^+$ T cells secrete some cytotoxic substances such as perforin present in the granules, which produce lesions in the membranes of target cells and lyse them.

3.1.3 Major histocompatibility complex in fish

The major histocompatibility complex (MHC) is a cluster of closely linked genes present in all vertebrate species. The genes of the MHC are inherited as a unit, a phenomenon known as linkage, i.e. they make a linkage between antigenic peptides and T lymphocytes. In fact, MHC genes encode essentially polymorphic cell surface glycoproteins which bind non-self peptides (degraded antigen) and present them to T lymphocytes (Townsend and Bodmer, 1989; Bjorkman and Parham, 1990) and thus initiate a specific immune response (Klein, 1986; Rothbard and Gefter, 1991).

To date, most of the work on MHC has been undertaken in homeothermic mammals and birds, and there is much less known about the system in lower vertebrates and invertebrates. Although phylogenetically, fishes are lower vertebrates, they possess the ability to mount a humoral immune response characterized by the induction of antigen specific immunoglobulins (Smith *et al.*, 1966; Dorson, 1981). Cellular alloreactivity can be observed through allograft rejection and mixed lymphocyte reactivity (Etlinger *et al.*, 1977; Rijkers, 1982). Teleost fish also appear to process functional lymphocyte subpopulations comparable to the T and B lymphocytes seen in higher vertebrate species (Secombes *et al.*, 1983; Miller *et al.*, 1986). These indirect

features of the immune system indicate the presence of a putative MHC homologue in fish but the evidence still remains conjectural.

In amphibians, for analysis of MHC, skin grafting between unrelated and related species has commonly been used. Cohen (1971) observed that urodelous amphibians typically rejected allografts slowly, which indicated the lack of an MHC complex, whereas anurous frog of the genus *Rana* characteristically exhibited acute allograft responses when appropriately challenged (Bovbjerg, 1966). The acute nature of allograft reactions among outbred frogs is seen as evidence for the existence of a major histocompatibility complex. Similar studies in teleosts also showed allografts to be rejected in an acute fashion (Borysenko, 1976; Botham *et al.*, 1980) and that a number of histocompatibility loci (4-7) were likely to be involved in this process in goldfish (Hildemann and Owen, 1956) and between 10-15 loci in different *Xiphophorus* species (Kallman, 1964).

Therefore, allograft rejection experiments in fish have served a dual purpose. Several scientists have used allograft experiments as a tool to investigate the developmental status of cellular immunity (Rijkers and Van Muiswinkel, 1977; Botham and Manning, 1981; Kikuchi and Egami, 1983), while others were more concerned with the genetics of tissue transplantation because there is a difference in the kinetics of graft rejection (reviewed in Hildemann, 1970; Kallman, 1970). Vertebrate animals are diploid and in nature mostly heterozygous at their MHC loci (Klein, 1986). The kinetics of graft rejection depends on the degree of heterozygosity at MHC loci in a given animal. In humans, the MHC complex is designated *HLA* which is located at four loci- *HLA-A*, *HLA-B*, *HLA-C*, and *HLA-D* and each of the four loci has a series of alleles. At present the HLA-B gene appears to be the most polymorphic with at least 32 distinct alleles and is followed by the HLA-A gene with at least 17 alleles. The other two genes,

HLA-C and *HLA-D* have 8 and 12 alleles respectively. In mouse, the MHC complex is known as *H-2* which has 4 loci, K, I, S, and D. At K locus more than 55 and at D locus 60 alleles have been identified (Zaleski *et al.*, 1983). Therefore in studies on the genetics of MHC, it is seen as very important to reduce the heterozygosity at MHC loci. In many species this can be done by producing inbred lines using gynogenesis or androgenesis or at least congenic lines. Du Pasquier *et al.* (1977) used gynogenesis in *Xenopus laevis* to provide evidence of the XLA complex, the MHC homologue in this species. Komen *et al.* (1990) observed that skin allografts exchanged among heterozygous gynogenetic siblings produced by meiotic gynogenesis survived for a longer period, whereas allografts exchanged among homozygous gynogenetic siblings produced by mitotic gynogenesis all rejected after a shorter period. Allografts on the clonal members derived from a homozygous female were fully accepted. Likewise, the related F_1 hybrids produced from the homozygous female by crossing with a homozygous male sibling accepted the grafts from the homozygous strain member, but the reverse grafts were rejected. Therefore, these results provide evidence for the idea that in carp histocompatibility genes exist, and that there is at least one major locus, and/or co-dominantly expressed multiple minor loci. In mice, rats, and humans, the major histocompatibility locus produces strong transplantation antigens which exert intense allograft reactions (Amos *et al.*, 1963; Elkins and Palm, 1966). But other minor histocompatibility loci from other chromosomes produce weak transplantation antigens that individually trigger relatively mild immune responses (Graff and Bailey, 1973, Hildemann, 1971).

Mixed lymphocyte reaction (MLR) experiments have been used to provide evidence for MHC genes in animals. In higher vertebrates, it is established that only MHC loci encoding class II molecules are responsible for MLR. The MLR have also

been observed in a number of fish species such as Atlantic salmon, *Salmo salar* (Smith and Braun-Nesje, 1982), rainbow trout, *O. mykiss* (Etlinger *et al.*, 1977), carp, *C. carpio* (Caspi and Avtalion, 1984; Grondel and Harmsen, 1984), and channel catfish, *Ictalurus punctatus* (Miller *et al.*, 1986). Among them, Caspi and Avtalion (1984) first described the usefulness of the technique for genetic analysis of MLR-recognised histocompatibility antigens in carp. In this study, the MLR responses between fish collected from different geographical locations were invariably medium to high, whereas fishes from a single source showed uniformly low responses which might be the consequence of some degree of inbreeding.

In recent years, molecular cloning techniques have facilitated the analysis of MHC in mammals. According to Klein (1986), MHC class II A and class II B genes have been cloned from a number of mammalian species. The mammalian class II molecules consist of one α and β chain and these two chains are encoded by separate class II A and class II B genes respectively. In the case of non-mammalian species, the MHC of the chicken (*Gallus gallus*) in which sequencing has identified classical class I (B-F), class II (B-L), and non-classical (B-G) genes (Bourlet *et al.*, 1988; Guillemot *et al.*, 1988). The genetic organisation of the MHC of chicken shows striking differences compared to that of mammals especially the distance between class I and class II molecules which are much shorter and interspersed with non-classical MHC genes (reviewed in Stet and Egberts, 1991).

To identify and isolate the MHC genes in an animal, cross hybridisation with homologous DNA probes already isolated from higher vertebrates can be used. In this way, the human MHC class II subprobe (HLA-DQβ) was successfully used to isolate the class II gene from chicken (Bourlet *et al.*, 1988) but in fish such attempts have generally been unsuccessful (reviewed by Kaufman *et. al.*, 1990; Stet and Egberts, 1991).

Recently, the introduction of the polymerase chain reaction (PCR) and nucleotide sequence techniques have accelerated the cloning of MHC genes from lower vertebrates which has led to both cDNA and genomic sequences from a variety of teleost fishes being obtained (Hashimoto *et al.*, 1990; Juul-Madsen *et al.*, 1992; Hordvik *et al.*, 1993; Ono *et al.*, 1992, 1993; Klein *et al.*, 1993) and cartilagenous fishes (Hashimoto *et al.*, 1992; Kasahara *et al.*, 1992). Hashimoto *et al.* (1990) first identified two putative MHC-antigen encoding sequences, TLAIα-1 and TLAIIβ-1 in carp, *C. carpio* which were homologous to both mammalian and avian MHC class I heavy chain and class II β chain respectively. The primers used for amplification of these sequences were synthesised from two highly conserved amino acid sequence blocks surrounding two cysteine residues in the second domain of MHC class II β chains as well as the third domain of class I heavy chains of human, mouse and chicken. Cloning of the β_2 - microglobulin gene has been used as an alternative possible means for identifying the teleostean MHC molecules because of its non-covalent association with class I molecules (Shalev *et al.*, 1981; Ono *et al.*, 1993). Recently it has been known that the organisation of fish MHC genes is quite different from mammalian MHC (Stet *et al.*, 1998). The similarity in amino acid sequences between fish and mammalian MHC molecules is relatively low, the maximum homology that has been found is only 40%.

Sequence data of MHC genes in fish has provided information on the level of polymorphism in a single locus. Klein *et al.* (1993) found extensive MHC variability in cichlid fishes of lake Malawi. They found high sequence variability of the MHC class II B genes in a sample and suggested that this variability can be used as a set of molecular markers for studying speciation during adaptive radiation. High levels of polymorphism in the MHC class II genes in teleosts have been reported by Stet *et al.* (1996) and

Langefors *et al.* (1997). Polymorphism of MHC genes can be used for stock identification and in the management of its improvement. Van der Zijpp and Egbert (1989) reported an association between MHC and disease in farm animals. If MHC polymorphism can be associated with disease resistance or susceptibility this information can be included in a selective breeding programme. In fact, it has been reported that several strains of fish differ in resistance or susceptibility (reviewed by Chevassus and Dorson, 1990).

In the present study, two different approaches were taken to determine the levels of MHC variation and effect in different clonal groups of tilapia, *O. niloticus*. Firstly, polymerase chain reaction (PCR) and secondly, scale grafting.

3.2 Polymerase chain reaction

The polymerase chain reaction (PCR) is one of the most useful techniques in molecular genetic studies. It is a selective DNA amplification method which can be used to amplify any desired part of the DNA from the whole. The greatest advantage of this method is that it is possible to amplify DNA from a very minute source of DNA, even from one cell. The availability of PCR has accelerated the cloning of MHC genes from lower vertebrates. Hashimoto *et al.* (1990) first cloned the MHC genes from carp by using PCR techniques.

The principles of PCR are that DNA polymerase uses single-stranded DNA as a template for the synthesis of a complementary new strand. These single-stranded DNA templates can be produced by simply heating double stranded DNA to a temperature near to boiling. DNA polymerase also requires a small section of double-stranded DNA to initiate synthesis (primer). Therefore, the starting point for DNA synthesis can be specified by using an oligonucleotide primer which anneals to the template at that point.

By this way, DNA polymerase can be directed to synthesise a specific part of the DNA molecule.

The resulting single-stranded DNA molecules can serve as templates for synthesising new complementary double-stranded DNA provided an oligonucleotide primer is supplied for each strand. The primers used are chosen to flank the region of DNA that is to be amplified so that the newly synthesised strands of DNA, starting at each primer, extend beyond the position of the primer on the opposite strand. As a result new primer binding sites are generated on each newly synthesised DNA strand. The reaction mixture is again heated to separate the original and newly synthesised strands, which are then available for further cycles of primer hybridisation, DNA synthesis, and strand separation.

Once started the PCR cycle can be repeated as many times as desired from 30 to 60 cycles. The net result of PCR is that by the end of n cycles, the reaction contains theoretically a maximum number of 2^n double-stranded DNA molecules that are copies of the DNA sequence between the primers (Watson *et al.*, 1992).

3.2.1 Materials and methods

To amplify the MHC class II B genes in *O. niloticus* by PCR, 10 ng of genomic DNA was used. The DNA was extracted mainly from blood and sometimes from fin samples. The blood samples were collected from caudal vein of fish by using sterile syringe and needles. The DNA was extracted by phenol and chloroform/ isoamyl alcohol extractions. The collection of blood and fin samples, and the procedures for extraction of total DNA from these samples have been previously explained in Section 2.2.16.1. After extraction the concentration of DNA was determined (Section 2.2.16.2)

and diluted in TE buffer in a total volume of 100 µl to give a final concentration of 10 ng μl^{-1}.

A total of 25 µl of PCR reaction mixture was prepared. Approximately 10 ng of total DNA was used as template for 25 µl of PCR reaction containing 10x buffer, 25 mM $MgCl_2$, dNTP's, two generic primers (TU 383 and TU 377), Red hot polymearse (5 units μl^{-1}) and sterile deionized water. The Red hot DNA polymerase (Advanced Biotechnologies Ltd, Surrey, UK) was used as the enzyme for strand extension in the PCR protocol. It has a 35 mins half life at 95 °C and can amplify up to 12.5 kb of DNA. The Red hot polymerase was used in the PCR mixture with reaction buffer [200 mM $(NH_4)_2SO_4$, 750mM Tris-HCl, pH 9.0, 1% (w/v) Tween], and 2mM $MgCl_2$ which was found optimum concentration for both primers and gave specific and strong PCR products. Two primers (originated from cDNA sequence of cichlid) employed for amplification of Intron 1 and Exon 2 of MHC class II B genes were provided by Edward Malaga and Jan Klein from the Max-Planck-Institut fur Biologie, Tubingen, Germany. The sequences of these primers are as follows

TU 383 - 5′ - CTC TTC ATC AGC CTC AGC ACA-3′

TU 377 - 5′ - TGA TTT AGA CAG A(G/A) (T/G)G (T/G)(T/C) GCT GTA -3′

The PCR reaction mixture was prepared in an individual 0.5 ml PCR microfuge tube. Therefore, during working with multiple samples with the same primers, a master

mixture was prepared, spun and the appropriate amount aliquoted into 0.5 ml individual microfuge tubes. After that 1µl (10 ng) of individual DNA was added to the tubes to give a total mixture volume of 25 µl. Finally, one drop of sterile mineral oil was added to the tubes. The tubes were briefly spun to mix the reaction ingredients and layer the mineral oil over the mixture to prevent the evaporation of the reaction mixture during the amplification process.

The following ingredients were used to make 25 µl of PCR reaction in each tube.

Reagents	Volume	Final concentration
10x reaction buffer	2.5 µl	1x
10 mM dNTP mix	0.5 µl	0.2 mM
25 mM $MgCl_2$	2 µl	2 mM
Forward primer	1.25 µl	1 µM
Reverse primer	1.25 µl	1 µM
Red hot polymerase	0.125 µl	0.625 unit
Sterile deionized water	16.38 µl	

The PCR reaction was carried out by placing the tubes in a DNA thermocycler (HYBAID Ltd. Middlesex, UK) and amplified with the following programme.

Template denaturation was accomplished at 94 °C for 1 min and then PCR amplification step for 40 cycles were programmed as follows:

Denaturation	94 °C	1 min
Annealing	55 °C	1 min
Extension	72 °C	2 mins

At the end of the amplification cycles, 10 mins for final extension was included to ensure completion of the reactions.

The amplified PCR products were then examined by electrophoresis in a 2% agarose gel along with a PCR marker (Sigma-Aldrich Co. Ltd.). The electrophoresis was carried out with 1x TBE buffer and the gel run at 5 V cm^{-1} for 3 hrs. Following electrophoresis, the PCR products were visualised and the position of the bands measured and compared with the PCR marker following the procedures as described in Section 2.2.17.1. The MHC class II B genes of ordinary broods, meiotic and mitotic gynogenetics, inbred and outbred clones from different families of *O. niloticus* were amplified.

3.2.2 Results

In the amplification of MHC class II B genes in *O. niloticus*, the schedule for PCR reaction was very effective. Two generic primers TU 383 (forward) and TU 377 (reverse) were designed to amplify the intron 1 and exon 2 of MHC class II B. The size of the intron 1 is variable in different families and can be used as a polymorphic marker. In Figure 3.2, the amplification of MHC class II B genes in broodstock demonstrated different patterns of bands. All of the lanes showed multiple bands, because the primers were generic and amplified several MHC loci, each of which has a defined length and sequence. The banding patterns were compared to each other and classified into different groups. Most of the bands were positioned between 300 and 750 base pairs. The sequencing of the bands generated a number of MHC class II B loci (Edward Malaga, personal communication) which are presented in Table 3.1. In some cases, some of the bands are non-MHC specific, that is, they are produced from unspecified PCR products.

The PCR amplification of MHC class II B genes in meiotic gynogenetics, mitotic gynogenetics and related diploid controls revealed that all gynogenetic offspring inherited only their maternal MHC class II B genes and there was no paternal inheritance in any of the gynogenetic groups (Figure 3.3 and Figure 3.4). Like DNA fingerprinting, the analysis of PCR products showed that in meiogynes a high percentage of recombination took place between chromatids resulting in every individual being heterozygous. On the other hand, the mitogynes were completely homozygous and their homozygosity resulted from fixation of one or other maternal allele. The diploid controls shared bands from both parents. After grouping of broods and their mitotic offspring on their respective MHC genotypes, a number of mature

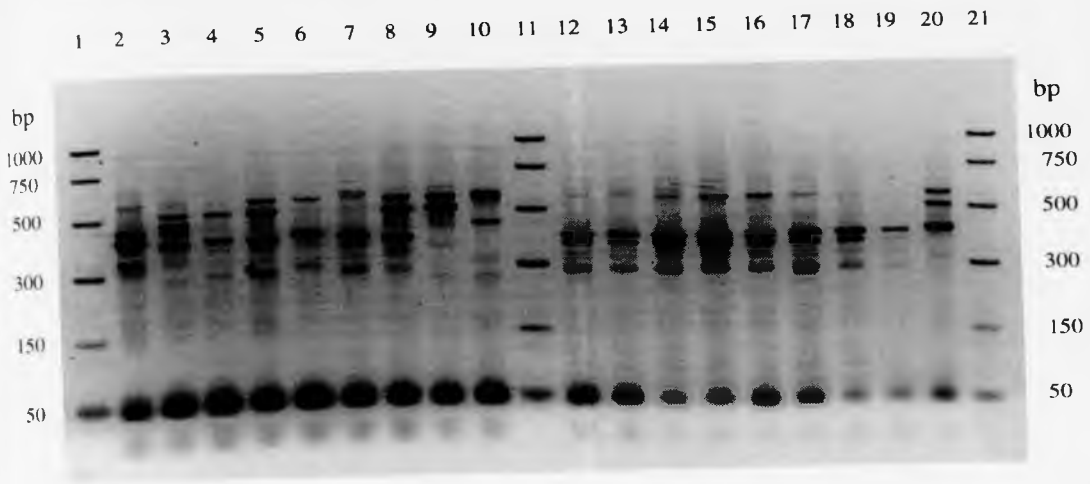


Figure 3.2 PCR amplification of MHC class II B genes of broodstock of *O. niloticus* with the TU 383 and TU 377 primers

Key: Lanes 2-10 and lanes 12-20 are different male and female broodstock. PCR marker fragments (bp) are positioned in lanes 1, 11 and 21

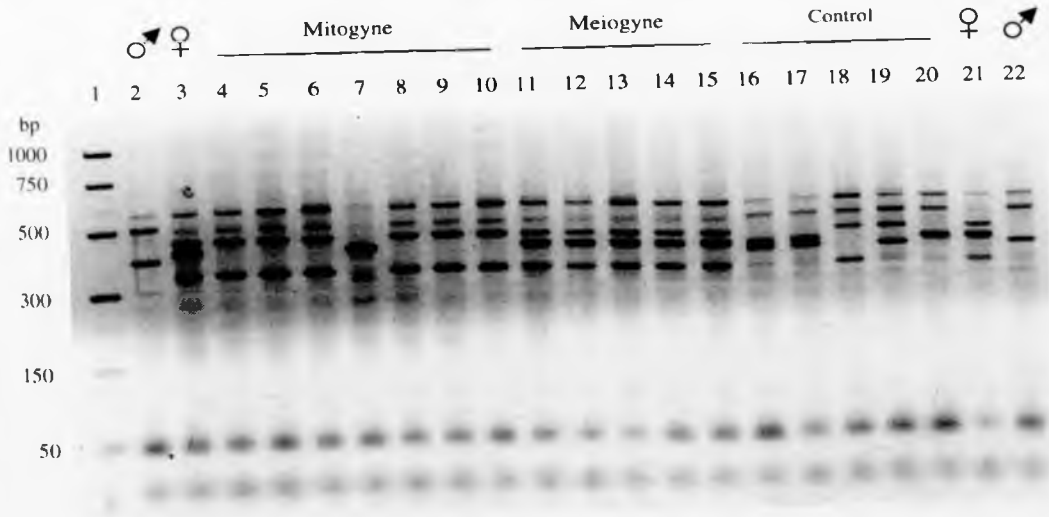


Figure 3.3 PCR amplification of MHC class II B genes of gynogenetic tilapia, *O. niloticus* with the TU 383 and TU 377 primers

Key: Lanes 2-22 father (009 037 284), lanes 3-21 mother (010 829 552), lanes 4-10 mitogynes, lanes 11-15 meiogynes and lanes 16-20 diploid controls. PCR marker is positioned in lane 1

Table 3.1 MHC class II B genotypes of female and male broodstock of *O. niloticus* and segregation of the genotypes in the subsequent inbred generations

Serial no.	Female and male broodstock		Mitogynes		Clones
	Tag no.	MHC class II B genotype (loci)	Tag no	MHC class II B genotype (loci)	MHC class II B genotype (loci)
2	010 829 552 ♀	(*4, 12**)	010 805 638	(*4**)	(*4**)
			010 842 049	(*12**)	-
3	000 863 059 ♂	(*1, 15, 2, 6**)	-	-	-
4	000 005 529 ♀	(*1, 12, 9**)	041 625 118	Unknown	-
5	009 120 547 ♀	(*1, 12, 9**)	001 019 320	(*1**)	(*1**)
			010 036 092	(*12, 9**)	(*12, 9**)
6	000 863 847 ♀	Unknown	006 812 566	Unknown	Unknown
			006 526 105	Unknown	-
7	000 770 303 ♀	(*6, 9**)	009 356 316	(*6, 9**)	(*6, 9**)
			009 819 347	(*6, 9**)	(*6, 9**)
8	009 037 284 ♂	(*1, 12, 9**)	-	-	-
9	000 112 323 ♀	(*1**)	000 886 064	(*1**)	(*1**)
			010 825 808	(*1**)	-
10	009 630 039 ♂	Unknown	-	-	-
12	000 291 557 ♀	(*6, 9**)	009 052 859	(*6, 9**)	-
			009 282 095	(*6, 9**)	-
13	000 367 361 ♀	(*6, 9**)	-	-	-
14	000 634 334 ♀	(*6, 9**)	-	-	-
15	000 362 121 ♂	(*1, 12, 9**)	-	-	-
16	000 786 087 ♀ (Androgenetic)	?			?
17	002 046 539 ♀ (Mitogyne)	(*6, 9**)			(*6, 9**)
18	002 041 887 ♀ (Mitogyne)	(*6, 9**)			(*6, 9**)
19	000 291 551 ♀	(*6, 9**)	002 046 539 ♀	(*6, 9**)	(*6, 9**)
			002 041 887 ♀	(*6, 9**)	(*6, 9**)
20	000 107 314 ♂	(*1, 12, 9**)	-	-	-

Serial numbers correspond to the broodstock with identical lane numbers in Figure 3.2

- No progeny produced

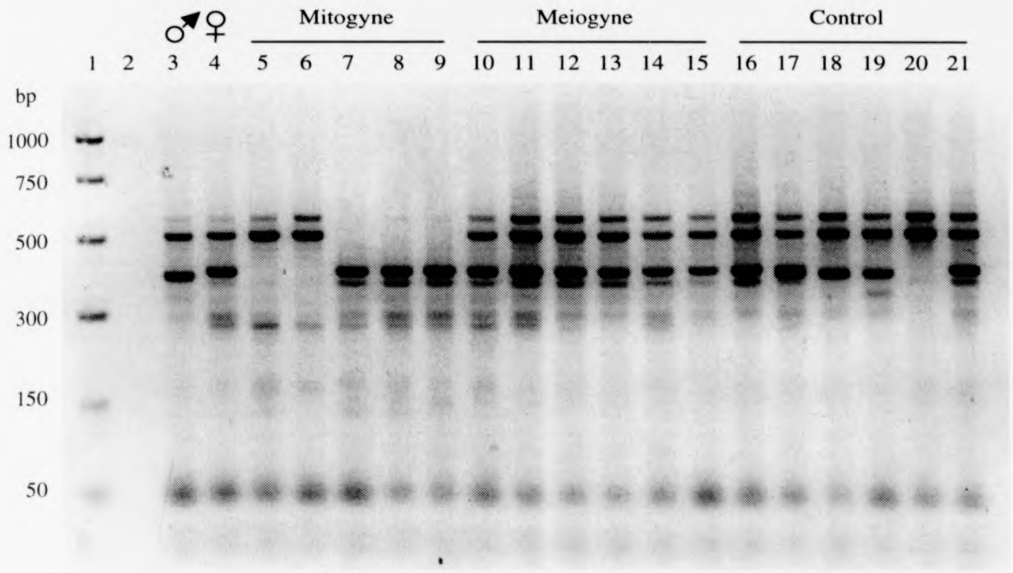


Figure 3.4 PCR amplification of MHC class II B genes of gynogenetic tilapia, *O. niloticus* with the TU 383 and TU 377 primers

Key: Lane 3-father (009 037 284), lane-4-mother (009 120 547), lanes 5-9 mitogynes, lanes 10-15 meiogynes and lanes 16-21 diploid controls. PCR marker fragments (bp) are positioned in lane 1

mitotic females were used to produce clones. Figure 3.5 showed that all of the clonal offspring and their mitotic mother have identical bands and none of the bands segregated in the offspring from their father. In the outbred clones, an identical banding pattern was found in all the progenies and their MHC inheritance occurred from both parents in a Mendelian fashion (Figure 3.6).

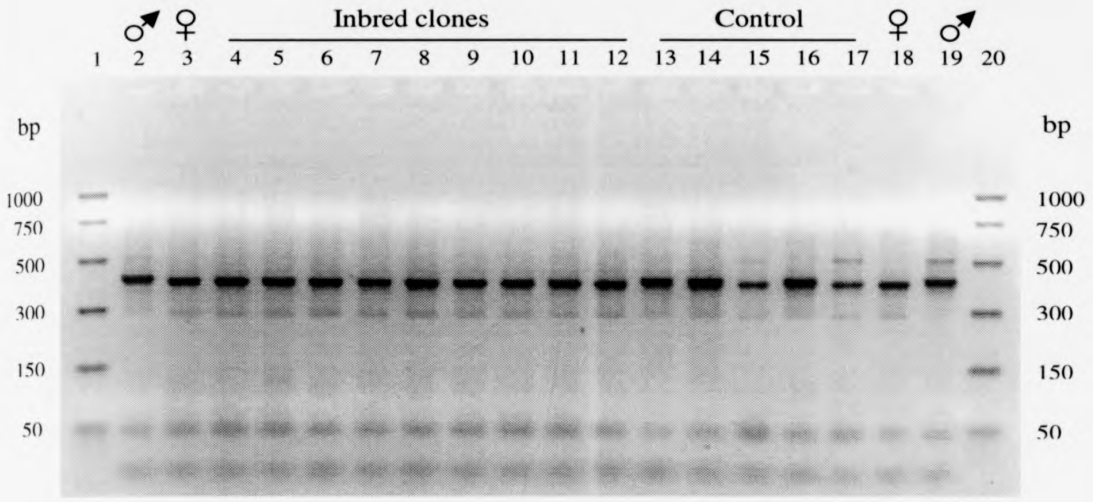


Figure 3.5 PCR amplification of MHC class II B genes of gynogenetic inbred clones in *O. niloticus* with the TU383 and TU 377 primers

Key : Lanes 2, 19- father (000 362 121), lanes 3, 18- mother (010 036 092), lanes 4-12 - inbred clones and lanes 13-17- diploid controls. PCR marker fragments (bp) are positioned in lanes 1 and 20

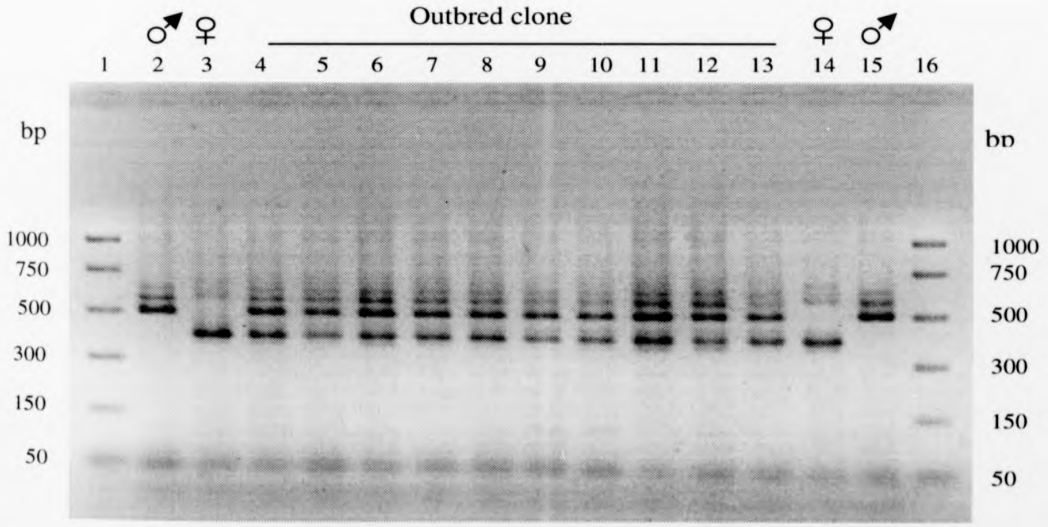


Figure 3.6 PCR amplification of MHC class II B genes of outbred clones in *O. niloticus* with the TU 383 and TU 377 primers

Key : Lanes 2, 15- father, lanes 3, 14- mother, lanes 4-13- outbred clones. PCR marker fragments (bp) are positioned in lanes 1 and 16

3.3 Scale grafting

Scale grafting has become the conventional method for determining the presence of the major histocompatibility complex in fish. When grafts are exchanged between genetically unrelated species, rapid graft rejection is often seen (Komen *et al.,* 1990). The rejection of allografts indicates that the individuals are incompatible with each other with respect to their major histocompatiblility complex. The various antigens that determine histocompatibility are encoded by more than 40 different loci in human, but the loci responsible for the most vigorous allograft-rejection reactions are located within the MHC. Identity of donor and host MHC genotype is not the sole factor determining tissue acceptance. When tissue is exchanged between genetically different individuals, even if their MHC antigens are identical, the transplanted tissue is likely to be rejected because of differences at a variety of minor histocompatibility loci. Unlike the major histocompatibility antigens, which are recognized directly by T_H and T_C cells, minor histocompatibility antigens are recognized only when they are presented in the context of self-MHC molecules. The tissue rejection induced by minor histocompatibility differences is usually less vigorous than that induced by major histocompatibility differences (Kuby, 1997).

Most of the studies on the immune response to foreign tissue in fish have involved the use of either skin or scale transplantation. Scale allograft rejection in many species of teleost has been reported (Hildemann, 1957; Hildemann and Hass, 1960; Triplett and Barrymore, 1960; Boryesenko and Hildemann, 1969; Avtalion *et. al.,* 1988). The process of scale grafting involves removing a scale from the epithelial pocket of one fish and inserting it into an empty pocket of another fish from which a scale has been removed (Mori, 1931, cited by Hildemann, 1957). The survival time of the scale allografts is measured from the time of transplantation to the time when all

melanophores within the scale have been destroyed. Alternatively, the growing of hyperplastic host tissue over the grafts can also be considered as the rejection end point (Rijkers and Van Muiswinkel, 1977). After transplantation of skin or scale allografts, the eventual rejection events are basically similar in all species of fish. Externally the allografts placed on a host fish can't be distinguished from autografts for the first few days. Then autografts usually completely heal within a few days, whereas allografts exhibit haemorrhage and begin to lose their pigmentation as melanophores are destroyed.

Histologically, the host response to an autograft differs markedly from that to allografts. The initial response is an invasion of lymphocytes and macrophages in the allografts. This does not occur in the autografts and appears to be common to all fish groups studied (Borysenko and Hildemann, 1969; McKinney *et al.*, 1981). Cellular infiltration into allografts begins early in the response and its peak proceeds the cytotoxicity events that result in melanophore breakdown and pigment dispersal (Botham *et al.*, 1980).

In the case of second-set allografts from the same donor, the rejection of grafts shows a similar pattern to that of the first-set graft, but the rate of rejection is comparatively faster with the second-set (Hildemann and Hass, 1960).

The mean survival time of allograft varies from species to species. It even varies between different skin or scale types and on the chosen end points for graft survival. For instance, in holostean fish with ganoid scales, allograft transplantation presents a different situation from that in teleost (McKinney *et al.*, 1981). Even within the teleosts, longer mean survival times are recorded for skin grafts compared to scale grafts. Physical factors such as temperature (Hildemann, 1957) and day-night rhythms may also influence immune reactivity (Nevid and Meier, 1993). Komen *et al.* (1990)

mentioned that the degree of genetic variability between animals might have influence on mean survival time of grafts. However, in more primitive groups of fish allograft rejection is reportedly slow (Hildemann, 1970). In agnathans, elasmobranches, and primitive actinopterygian fish, chronic graft rejections were found with first-set grafts surviving for several weeks (>30 days). In contrast, teleost fish reject allografts more rapidly in an acute manner and this characteristic is usually associated with the presence of MHC genes.

3.3.1 Materials and methods

The scale transplantation was carried out following the methods as described by Hildemann (1957). For reciprocal scale grafting, selected fish were transferred to Perspex tanks in a UV-sterilized recirculating water system. The temperature of water was maintained at 28 ±1 °C. At least two weeks before grafting, the fish were transferred to the tanks so they could become acclimatized.

The donor and recipient fishes were anaesthesized with benzocaine as described in Section 2.2.2 and were placed side by side on a thin wet sponge. A 90 mm Petri dish containing 0.8% saline was used to hold one scale briefly during reciprocal grafts exchange. The scale grafting was performed by inserting a foreign scale into an empty pocket from which an autologous scale has been removed a few minutes earlier. Eight scales were reciprocally transplanted between paired fishes on one side and another six autologous scales grafted on the other side. For convenience, grafts were made in the row of scales just above or below the lateral line, using alternate scale position numbered from the operculum. To ensure complete adherence of grafts and to prevent infection, a small amount of Orahesive powder was spread around the base of the grafts. Following grafting, the fishes were transferred carefully back into their original tank.

The grafts were checked every day by observing the fish through the side of the transparent Perspex aquarium. Sometimes it was very difficult to check all the grafted scales due to the movement of the fish. Once a week the fish were anaethesized in their tank by benzocaine and the grafts checked more accurately. The progress of graft rejection was determined by observing the following events: haemorrhage (considered as first sign of rejection), hyperplasia and loss of pigmentation. During checking any rejected scales were removed and examined under microscope. Three weeks after grafting, a number of allografts and normal scales were sampled from different groups of fish and preserved in fixatives for histological study. The survival end point of the grafts was determined as the number of days after transplantation at which all allografts were rejected.

For reciprocal scale grafting, three clonal lines based on different MHC class II B genotypes were selected. Since the MHC class II genes are segregated to the offspring as haplotypes, the genotypes of the clonal lines represent certain MHC class II B haplotypes. The genotypes of the clonal lines were *(6,9*)*, *(12,9*)* and *(1*)* and these lines were identified with their mitotic mothers tag numbers, 009 356 316, 010 036 092 and 000 886 064 respectively. A total of 12 perspex tanks (30L water capacity) each of which was divided into two sections with perspex partitions were used. For grafting, three combined reciprocal groups [009 356 316*(6,9*)* ⇔ 010 036 092*(12,9*)*, 010 036 092*(12,9*)* ⇔ 000 886 064*(1*)*, 000 886 064*(1*)* ⇔ 009 356 316 *(6,9*)*] were made among the three clonal lines and each group of fish maintained in four tanks with four reciprocal pairs. Fish of a similar size and age were selected from the three lines and transferred to the tanks. Each of the reciprocal fish received two sets of allografts (8 scales in one set) and autografts (6 scales in one set), and three weeks after the rejection of first set, the second set of grafts were transplanted.

3.3.1.1 Histological examination of scale grafts

Both normal and allograft scales (10 for each type) were collected from fish and half were preserved in 10% neutral buffered formalin and half in Bouin's solution. This was to examine the effects of different fixatives on the scales. The scales were kept in buffered formalin for at least 24 hrs before processing. After 8-10 hrs in Bouin's fixative, the scales were first rinsed with, and then left for over night in 70% ethanol. The following day the scales were rinsed again with 70% ethanol before being processed.

3.3.1.1.1 Scale processing

The preserved scales were placed into coded cassettes, two similar scales per cassette. After cassetting, scales preserved by buffered formalin were kept in a small bowl full of water, and scales from Bouin's solution were kept in absolute alcohol until loading on to an automatic tissue processor (Shandon Citadel 2000). The autoprocessing steps were organized in such a way that the tissues pass through different alcohol grades, followed by absolute alcohol, chloroform and finally impregnation in molten wax. The detailed tissue processing schedule is presented in Appendix 3.1. After processing it was found that the scales preserved in Bouin's solution remained normal, while those preserved in buffered formalin became twisted. However, during sectioning both types of scales gave good sections.

3.3.1.1.2 Embedding

At the end of processing, the cassettes were removed from the processor and placed in the auxiliary wax bath on the histoembedder. The scales were removed from the cassettes, embedded and blocked in suitable sized moulds with paraffin wax (50-60

°C) and cooled rapidly by placing the moulds on a cool plate. Approximately 5 mins after the paraffin become solidified, the blocks were removed from the base mould and were stored at room temperature until sectioning.

3.3.1.1.3 Sectioning of tissues

The surface layer of the blocked wax was removed by trimming to expose the complete surface of the tissue. This was carried out by using a Leica 2035 BIOCUT microtome and an old surgipath disposable blade. Since scales are considered a hard tissue they were decalcified by placing the trimmed blocks face down in a vessel containing a layer of rapid decalcifying solution for at least 1 h. After that the blocks were washed with water, clamped into the block holder and sectioned to a thickness of 10 µm using new microtome blades. The ribbon of the sections was floated on a water bath at 40 °C and the best section was picked up on a clean glass slide. The slides were then marked using a diamond pen and dried in an oven at 60 °C for few hours before they were stained.

The slides with tissue sections were stained with haematoxylin and eosin stain. Details of the haematoxylin and eosin staining method is given in Appendix 3.2. Following staining the slides were washed in xylene for 5 mins and finally mounted with DPX (BDH).

3.3.2 Determination of mean survival time (MST-50)

During observation of grafted scales it was found that simultaneous graft rejection did not occur either in the different groups nor even on an individual fish. Therefore, a mean survival time for 50% of the transplanted scales for each fish had to be individually determined by plotting a cumulative scale rejection curve against time in

days. Then the mean survival time of 50% of scales (MST-50) of the individual group was determined. The differences in the mean survival times (MST-50) of both first and second set of allografts for recipient and donor groups were analyzed by one way ANOVA at the significant level of $P<0.05$. The differences in the MST between first and second set of allografts for each recipient and donor were analyzed by using Student's t-test at the same significant level as above.

3.3.3 Results

The observations of grafted scales showed that there was a clear pattern of progression; 2-3 days after grafting scales became reddish and seemed to have reestablished their capillary circulation. After this period all autografts became well healed within 6-7 days with no signs of transplantation in the autografted area. In contrast, most of the allografts became more reddish and distinctive from surrounding normal scales. Inflammation and slight haemorrhage was observed in some scales before rejection.

In the first set of allografts, the inflammation was observed at 5-6 days after transplantation and their rejection started at about 7 days. Fish from all groups showed slow rejection of grafts and by 42 days, about one-fifth of all fish had rejected all their grafts and the rest of them had rejected between 80-90% (Figures 3.7, 3.8 and 3.9). The

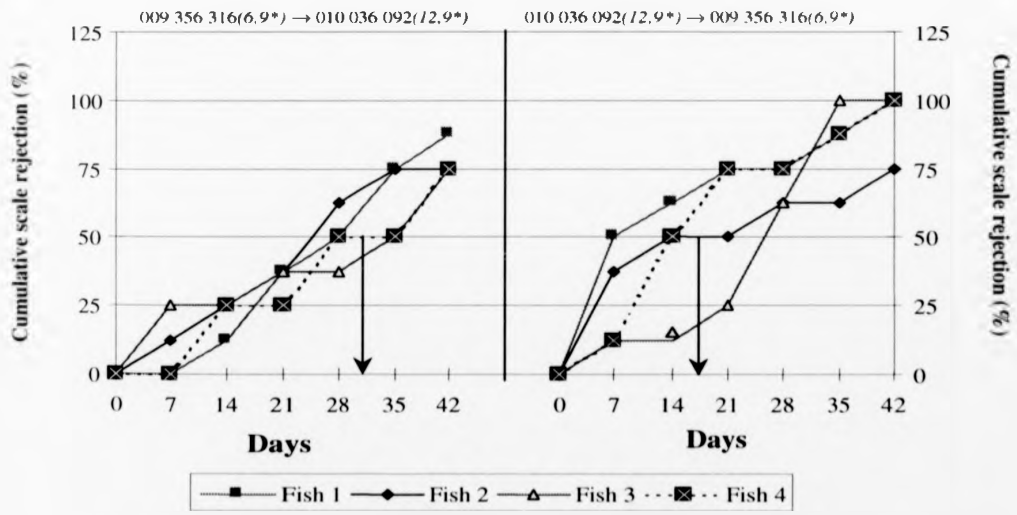


Figure 3.7 First set of scale allograft rejection profiles between two reciprocal groups [009 356 316(6,9*) ↔ 010 036 092(12,9*)] of cloned tilapia, *O. niloticus*

Arrow indicates the group mean survival time of grafts

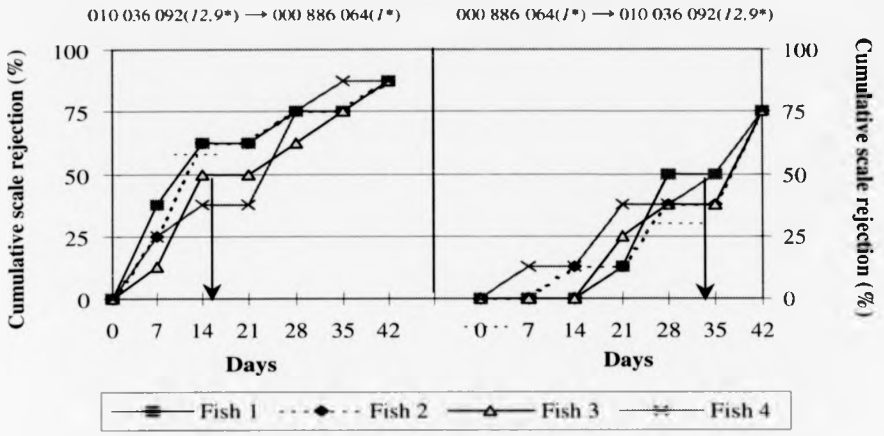


Figure 3.8 First set of scale allograft rejection profiles between two reciprocal groups [010 036 092*(12,9*)*↔000 886 064*(1*)*] of cloned tilapia, *O. niloticus*

Arrow indicates the group mean survival time of grafts

second sets of allografts were rejected significantly ($P<0.05$) faster than the first sets and in an acute manner. Severe inflammation and haemorrhage were observed in most of the grafts 2-3 days after transplantation and most of the fish rejected more than 50% of their grafts within 7 days. Complete graft rejection had occurred in all the groups between 21 and 28 days after transplantation (Figures 3.10, 3.11 and 3.12).

The MST of both first and second sets of allografts for donors and recipients are presented in Table 3.2. For the first set of grafts, all recipient groups of fish showed chronic rejection. The MST of grafts in the recipient 010 036 092*(12,9*)* was significantly longer ($P<0.05$) than those of other two recipient 009 356 316*(6,9*)* and 000 886 064*(1*)* groups. In the case of donors, in contrast, the MSTs of grafts in the 009 356 316*(6,9*)* and 000 886 064*(1*)* groups were significantly longer ($P<0.05$) than that of the 010 036 092*(12,9*)* group but they were not significantly different from each other.

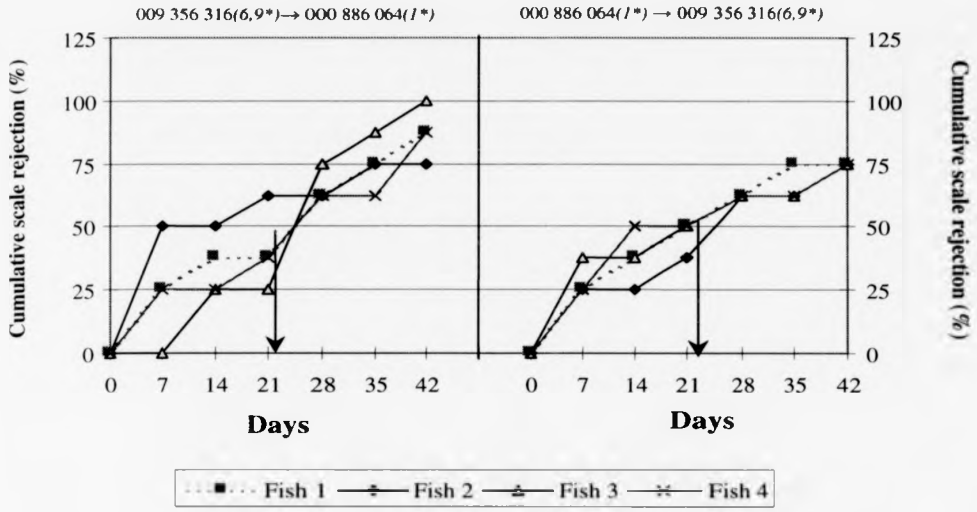


Figure 3.9 First set of scale allograft rejection profiles between two reciprocal groups [009 356 316*(6,9*)* ↔ 000 886 064*(1*)*] of cloned tilapia, *O. niloticus*

Arrow indicates the group mean survival time of grafts

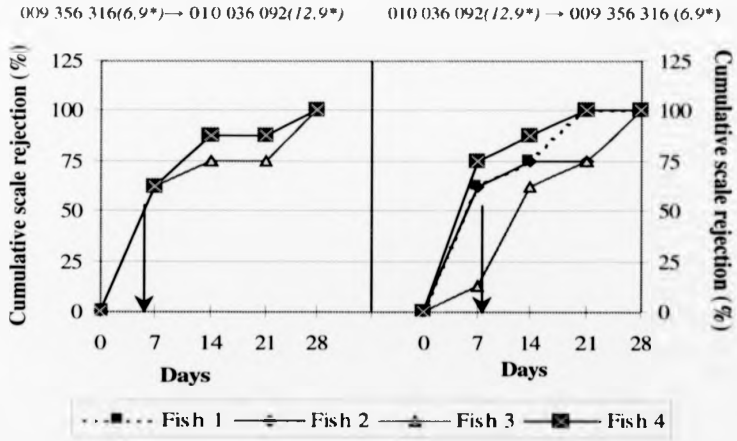


Figure 3.10 Second set of scale allograft rejection profiles between two reciprocal groups [009 356 316*(6,9*)*↔ 010 036 092*(12,9*)*] of cloned tilapia, *O. niloticus*

Arrow indicates the group mean survival time of grafts

The second sets of grafts were acutely rejected by all the fish groups in a similar manner and the MSTs of grafts were significantly shorter ($P<0.05$) than those of the first sets. The MSTs of the second set of grafts for either recipients or donors did not show any significant difference.

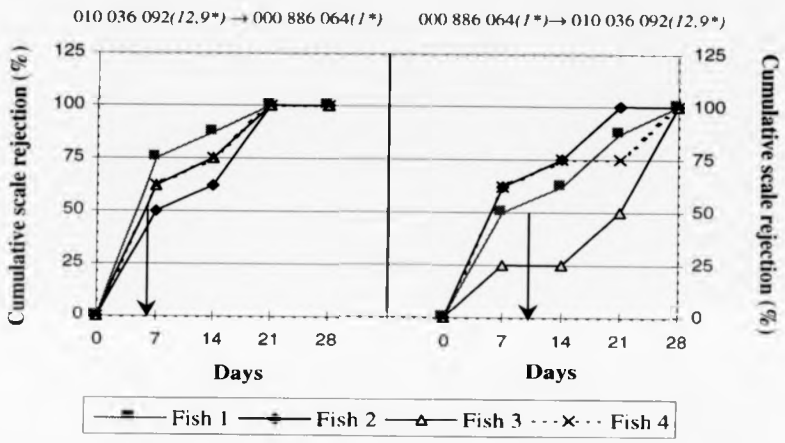


Figure 3.11 Second set of scale allograft rejection profiles between two reciprocal groups [010 036 092*(12,9*)*↔ 000 886 064*(1*)*] of cloned tilapia, *O. niloticus*

Arrow indicates the group mean survival time of grafts

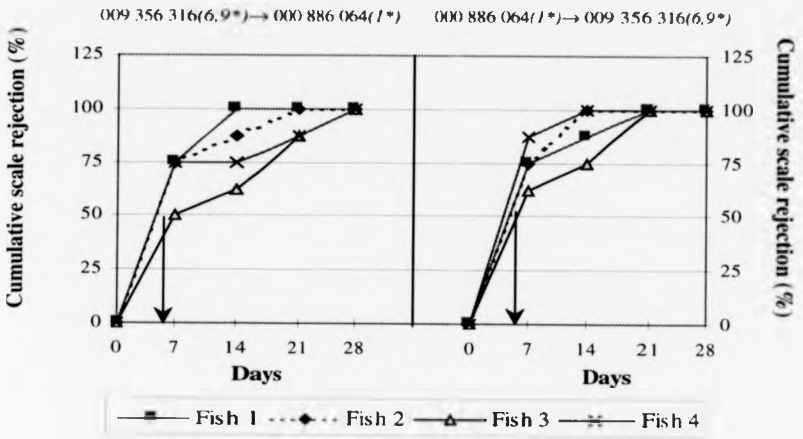


Figure 3.12 Second set of scale allograft rejection profiles between two reciprocal groups [009 356 316*(6,9*)*↔000 886 064*(1**)] of cloned tilapia, *O. niloticus*

Arrow indicates the group mean survival time of grafts

The histological examination of grafts showed some signs of histoincompatibility in the allograft scales. The histological sections of normal scales (non-transplanted) showed an even distribution of melanophores in the epidermal region and a thick epithelium with secretory cells, lying on a uniform scale plate (Figure 3.13). Allograft sections in contrast showed few broken melanophores in the dermal region and a large number of lymphoid cells clustered at the soft tissue scale plate junction (Figure 3.14).

Table 3.2 Scale allograft rejection and mean survival time (MST-50) of first and second set of grafts in different clonal groups of tilapia in *O. niloticus*

Donor →		009 356 316(*6,9**)[a]		010 036 092(*12,9**)[b]		000 886 064(*1**)[a]	
Recipient ↓		1st set	2nd set*	1st set	2nd set*	1st set	2nd set*
009 356 316(*6,9**)[p]	MST ± S.E.	0	0	17.0 ± 4.14	7.25 ± 1.60	21.75 ± 0.75	5.0 ± 0.41
	No. of fish	4	4	4	4	4	4
	No. of scales grafted	24	24	32	32	32	32
	No. of scales rejected	0	0	30	32	24	32
010 036 092(*12,9**)[q]	MST ± S.E.	30.75 ± 2.5	6.0 ± 0	0	0	34.25 ± 2.14	10.25 ± 3.59
	No. of fish	4	2[#]	4	4	4	4
	No. of scales grafted	32	16	24	24	32	32
	No. of scales rejected	25	16	0	0	24	32
000 886 064(*1**)[p]	MST ± S.E.	21.5 ± 2.5	5.5 ± 0.50	14.75 ± 3.20	6.0 ± 0.41	0	0
	No. of fish	4	4	4	4	4	4
	No. of scales grafted	32	32	32	32	24	24
	No. of scales rejected	28	32	28	32	0	0

After second set allograft transplantation, two fish died from 009 356 316 group

Donors and recipients with different letters (a, b) and (p, q) respectively indicate significant difference ($P<0.05$) for the MST of their first set of allografts

* MST of the second set of allografts in both donors and recipients significantly different ($P<0.05$) from respective first set of allografts

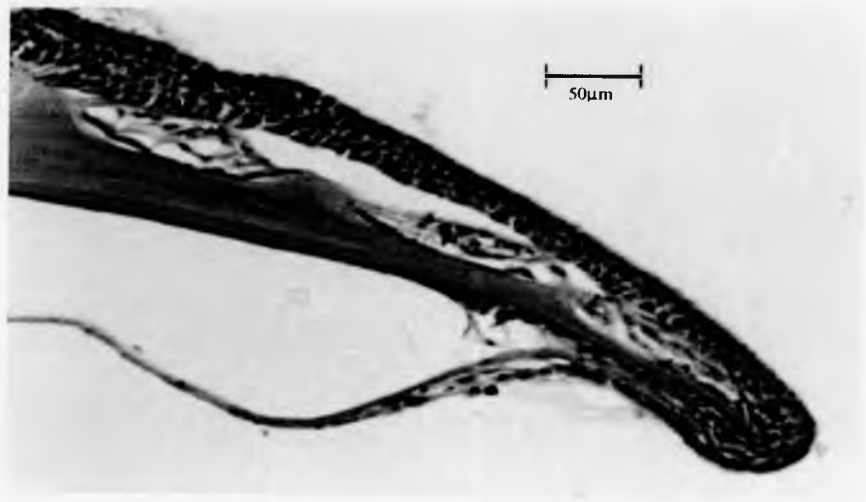


Figure 3.13 Histological section of a normal scale of *O. niloticus*. The transverse section shows an even distribution of melanophores and thick epidermis with secretory cells, lying on a uniform collagenous scale plate

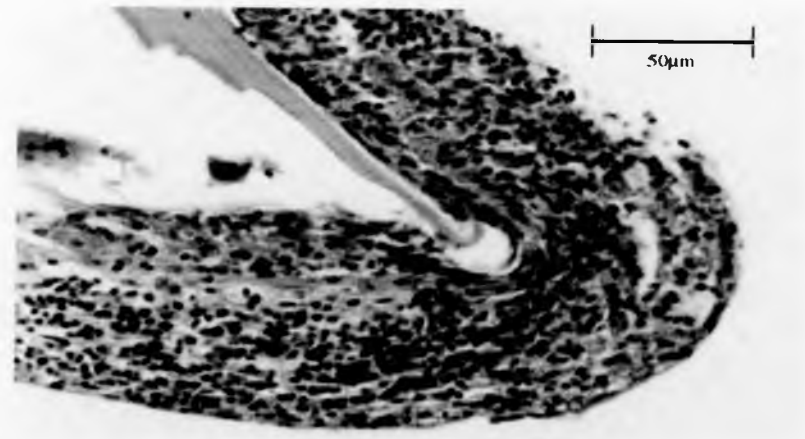


Figure 3.14 Histological section of an allograft scale of *O. niloticus*. The transverse section shows lack of intact melanophores and the presence of numerous lymphoid cells in the epidermal and dermal regions

3.4 Discussion

The presence of MHC class II B genes in *O. niloticus* was successfully determined by PCR amplification using two generic primers. The MHC class II genes are polymorphic in nature and might be located in multiple loci. At least 17 polymorphic loci have been identified in cichlids and the number of loci varied from individual to individual, ranging from 1 to 13 (Malaga *et al.*, in preparation). In the broodstock of *O. niloticus*, the two generic primers amplified MHC class II genes from multiple loci which resulted in multiple bands in each individual fish. The number and position of the bands were different from each other and the pattern of bands made them individual-specific for MHC class II B loci (Figure 3.2 and Table 3.1).

The amplification of MHC class II genes in gynogenetic fish and their clonal offspring revealed that this technique might be effectively used in the analysis of chromosome manipulation experiments. It was evident that like *ADA** loci, MHC loci from mitotic gynogenetic mothers segregated in their clonal offspring. The segregation pattern of MHC loci and their polymorphism facilitated to group mitotic females for the production of clonal lines. For example, in Table 3.1 the MHC class II B genotype of the female 010 829 552 is (*4,12**). During the production of mitotic gynogenetics from this female, these two loci segregated in the mitogynes and as a result some of the mitogynes have (*4**) genotype and some of them have (*12**). Similarly, the female 009 120 547 has (*1,12,9**) MHC class II genotype, which is segregated in the mitogynes as (*1**) and (*12,9**) genotypes. As the mitogynes are fully homozygous for every MHC locus and the number of loci depends on particular haplotypes (21 distinct haplotypes have been identified), clones produced from different mitotic females should be different. The number of loci varied in each haplotype, therefore the functional expression of MHC class II genes should also be different in each clonal line. Since the

expression of MHC haplotypes is closely related to disease resistance (Klein, 1986), the clonal lines can be used in immunological experiments.

In the present study, besides PCR, scale grafting was also used to determine MHC genes in *O. niloticus*. In scale grafting, reciprocal grafts will be accepted by two individuals if they are genetically compatible to each other, otherwise grafts will be rejected. Three clonal lines with different MHC class II B genotypes *(6,9*), (12,9*)* and *(1*)* were used. The first set of autografts and allografts in all the reciprocal groups were indistinguishable for the first 2 days after transplantation. The blood circulation was seemed to be restored and the grafts became slightly inflammed. The circulation in autografts had established permanently within 7 days but in allografts the capillarisation was slow and often incomplete. This phenomenon persisted for the first few days and after 5-6 days inflammation was observed in the allografts. Graft rejection started slowly in all the groups and continued for few weeks in a chronic manner. Although survival end point of grafts was determined by their complete rejection, sometimes in chronic condition the end point was determined by observing the generation of an autologous new scale. Since scale grafting was performed within three clonal groups of fish, each of the groups served dual functions: one was donor and another was recipient. Therefore, two MSTs were obtained from each group of fish for each set of grafts (1st and 2nd set graft). For the first set of grafts, the recipient 010 036 092*(12,9*)* showed significantly slower ($P<0.05$) rejection (MST >28 days) than the other two recipients. Slow allograft rejection profiles have been found in the F1 gynogenetic pairs of *O. aureus*, where graft rejection started 7 days after transplantation and continued to the end of 42 days (Avtalion *et al.*, 1988).

In the case of second set grafts, the rejection rates were significantly ($P<0.05$) faster than in the first set. After transplantation, there was generally no restoration of

capillary circulation and severe inflammation and haemorrhage were observed in most of the allografts and resulted in acute rejection. Although in the first set of grafts, slow rejection tendency was found in all the groups, the MST in the 010 036 092*(12,9*)* group for both donor and recipient levels showed a significant difference from other two 009 356 316*(6,9*)* and 000 886 064*(1*)* groups. The differences in MST of grafts between groups might be due to the effect of different MHC class II B genes where different kinds and numbers of MHC loci are involved. In the case of fast graft rejection, the particular MHC loci might be elicited strong alloantigenic effect against foreign grafts by presenting the foreign peptides to T lymphocytes through the surface of the antigen presenting cells. The recognition of alloantigens by T lymphocytes induces vigorous T-cell proliferation which might lead to faster graft rejection through CTL-mediated killing. Cohen and Hildemann (1968) and Graff *et al.* (1966) suggested that the differences in MST of grafts between groups might be related to the presence of strong versus weak histocompatibility genes. Komen *et al.* (1990) observed that a single set of skin grafts reciprocally transplanted between two homozygous clonal strains of common carp were completely rejected, and one strain rejected grafts slightly slower (MST 16.6 ± 3.3 days) than the other strain (MST 13.1 ± 3.3 days) but the difference of MST was not significant. From these results he suggested that the presumed strong histocompatibility (H) locus is polymorphic and exits in allelic forms of different strength, which has been found in mice and humans (Klein, 1982).

In the case of the second set allografts, positive rejection responses were observed in all the groups with a similar acute manner. Even the recipient group 010 036 092 (*12,9**) which had demonstrated comparatively the most chronic rejection for their first set allografts, also showed acute rejection of their second set grafts similar to other groups. The MST of the second sets of allografts in all the groups (recipient and

donor) were significantly ($P<0.05$) shorter than the first sets. The faster rejection response of fish to the second set grafts shows the specificity and immunological memory characteristic of the cell mediated immune response. Botham and Manning (1981) observed that at 22 °C, the MST of the first set of grafts in carps was 14 days and the second set was 7 days in *C. carpio*. A similar trend of graft rejection in *Osteoglossum bicirrhosum* has been reported with a MST of 17.9 ± 1.8 and 5.1 ± 1.1 for the first and second set grafts respectively (Boryesenko and Hildemann, 1969). Perey *et al.* (1968) reported that chrondronstean species, *Polyodon spatula* rejected first set skin allografts in a chronic manner at 65-77 °F and second set allografts much faster, even at a lower temperature of 42-55 °F. Hildemann (1957) observed shorter median survival time of second set scale homografts compared to the first set in goldfish (*Carassius auratus*).

The histological examination of allografts showed all the well known phenomena associated with cellular incompatibility found in other fish such as pigment cell destruction, invasion of lymphoid cells and digestion of the scale plate. The grafts for histological section were collected directly from fish after 21 days of transplantation. Although most of the rejected scales were collected and preserved in fixatives, they were not used for histology. Most rejected scales were broken and the scale plates were partially digested before being lost. Some rejected scales were stained by Giemsa solution following the procedures described by Avtalion *et al.* (1988). They reported successful observation of inflammatory changes in the stained grafts showing an intensive lymphoid infiltration and serious tissue degeneration, but the same approach was not successful in the present study. The histological sections of the sampled scales showed some remarkable changes in the allografts. The normal scale usually exhibits an evenly distributed layer of melanophores in the epidermis and a thick epithelium with

secretory cells on the scale plate. In contrast, the allografts showed a broken layer of melanophores and a massive intrusion of lymphocytes and macrophages. Similar histological observations were reported by Boryesenko and Hildemann (1969) in scale allografts of the primitive teleost, *Osteoglossum bicirrhosum*, and also by Hildemann (1957) in scale homotransplantion of goldfish, *Carassius auratus*.

In conclusion, it can be stated that *O. niloticus* possesses functional MHC class II B genes and the segregation of these genes in the subsequent generations takes place as haplotypes. The MHC loci can be determined by PCR amplification and sequencing techniques, and the polymorphism of these loci can be used as molecular markers. The rejection of allografts between clonal lines strongly argued the presence of different MHC class II genes and their involvement in the immune response reactions.

Chapter IV

Non-specific immune responses of *Oreochromis niloticus*

4. Non-specific immune responses of *Oreochromis niloticus*

4.1 Introduction

In the previous chapter, the specific immune response of different clonal groups of tilapia were examined by scale grafting. All reciprocally transplanted scales were acutely rejected by the recipient clonal groups. Here, variations in the non-specific immune response of different clonal groups are investigated, since non-specific immune responses can determine the disease susceptibility of a given group of fish to a bacterial challenge.

4.1.1 Fish immune response

Like mammals and other vertebrates, fish have both non-specific (innate) and specific (acquired) immune systems and they work together protecting the animal from invading pathogenic organisms. Functionally, an immune response can be divided into two inter-related activities, recognition and response. The immune system of fish has the capability of recognising foreign particles and distinguish them from self. When a foreign particle is recognised, both the non-specific and specific immune systems mount an appropriate response to eradicate or neutralise the particle.

4.1.2 Non-specific immune system

The non-specific immune system acts as a first line of defence against infectious pathogens and potential parasites. It has no specific recognition or memory and thus commonly responds to most invading agents. The non-specific immune system is comprised of humoral and cellular defence mechanisms. When a pathogen breaches the physical barriers and enters the body of the fish, the first line of defence encountered by the

organism is components of the non-specific response such as mucus, lysozyme, complement, C-reactive protein, transferrin, interferon, and cellular phagocytes.

4.1.2.1 Lysozyme

Lysozyme is a hydrolytic enzyme found in different organs of the fish and is able to cleave the peptidoglycan layer of bacterial walls. It occurs in a wide range of vertebrates (Osserman *et al.*, 1974) and acts against invading microorganisms. Lysozyme prevents the invasion of Gram-positive bacteria by splitting the β linkages between N-acetylmuramic acid and N-acetylglucosamine in the cell wall of bacteria (Salton and Ghuysen, 1959). Lysozyme has no direct action on Gram-negative bacteria, but once complement and other enzymes have destroyed the outer cell wall of the bacteria, it becomes effective in breaking the inner peptoglycan layer of the bacterium (Glynn, 1969; Neeman *et al.*, 1974; Hjelmeland *et al.*, 1983). As well as direct antibacterial activity, lysozyme also enhances phagocytosis by acting as an opsonin or by directly activating polymorphonuclear leucocytes and macrophages (Klockars and Roberts, 1976; Jolles and Jolles, 1984). Salton (1957) and Jolles (1969) defined "true" lysozyme as an enzyme which satisfied the following criteria : i) is able to lyse *Micrococcus lysodeikticus* cells, ii) is readily absorbed by chitin-coated cellulose, iii) is a low molecular weight protein, and iv) is stable at acidic pH and higher temperature, while inactivated at alkaline pH. The optimum activity of lysozyme is between pH 6-7. It has an isoelectric point between pH 10.5-11.0 and an approximate molecular weight of 14400 KD (Osserman *et al.*, 1974).

Lysozyme is widely distributed in nature and is found in animal secretions such as mucus, saliva and in many tissues including blood, and in the cell vacuoles of plants (Jolles,

1969). In fish, it is distributed mainly in leucocyte-enriched tissues, like the head kidney and other vulnerable sites for bacterial infection such as skin, gills, the alimentary tract, and in the eggs (Fletcher and White, 1973; Studnicka *et al.*, 1986; Grinde *et al.*, 1988; Yousif *et al.*, 1991; Holloway *et al.*, 1993). It has been reported that fish lysozyme plays an important role in the host defense system against infectious diseases (Fänge *et al.*, 1976; Murray and Fletcher, 1976; Lindsay, 1986; Lie *et al.*, 1989a).

Murray and Fletcher (1976) identified lysozyme activity in the monocytes and neutrophils of plaice by histochemistry. These cells probably contribute to serum lysozyme activity since their number increases concomitantly with serum lysozyme levels (Fletcher and White, 1973). Yousif *et al.* (1991) found high levels of lysozyme in the eggs of coho salmon and assumed that it was released from the kidney and other lysozyme-rich tissues of the mother and transported to the developing eggs through serum.

The lysozyme activity in fish is influenced by a variety of factors such as season, temperature, sex and sexual maturity. Fletcher *et al.* (1977) observed seasonal variations in the serum lysozyme concentration of lumpsuckers, and the concentration was higher in male fish than in females. Seasonal and sexual variations in serum lysozyme levels have also been reported for rainbow trout (Vladimirov, 1968). Fletcher and White (1976) observed a 70% decrease in serum lysozyme levels in plaice after being maintained at 5°C for 3 months. A similar decrease in serum lysozyme level was also observed in carp when maintained at low water temperatures (Studnicka *et al.*, 1986). However, the opposite result was found with Japanese eel. When they were maintained at 15 °C they had a higher serum lysozyme activity compared with those maintained at 20-30 °C (Kusuda and Kitadal, 1992). Mock and Peters (1990) reported that under stressful conditions like transport or

acute water pollution, serum lysozyme levels in rainbow trout decreased significantly. Enhanced serum lysozyme activity was observed in carp infected with *Aeromonas punctata* (Vladimirov, 1968; Siwicki and Studnicka, 1987) and in Atlantic salmon challenged with *A. salmonicida* (Moyner *et al.*, 1993).

4.1.2.2 Complement

The complement system is another essential part of the vertebrate immune response. The mammalian complement system is composed of two distinct pathways, the classical complement pathway (CCP) and the alternative complement pathway (ACP). The CCP is commonly initiated by the formation of soluble antigen-antibody complexes or by the binding of antibody to antigen on a suitable target such as a bacterial wall. On the other hand, ACP is initiated by various cell-surface constituents which are foreign to the host.

Fish, like mammals, have a distinct complement system, activated by both the CCP and ACP (Sakai, 1992). Most studies carried out relating to the complement system have been performed using mammals. Similar studies with fish have shown that many of the components of their complement system are similar to those of mammalian systems. The mammalian complement system is composed of nine components C1-C9 that participate in a controlled enzymatic cascade while killing a pathogen. Serum from Cyclostomes, lamprey and hag fish, lack cytolytic complement activity and are only activated via ACP (Fujii *et al.*, 1992; Nonaka, 1994). Two proteins have been isolated from lamprey, *Lampetra japonica* (Nonaka *et al.*, 1984a) and hag fish, *Eptatrutus burgeri* (Fujii *et al.*, 1992; Ishiguro *et al.*, 1992) and both are homologous to mammalian C3. The complement system of Elasmobranchs consists of both CCP and ACP. Its CCP is composed of six

functionally distinct components. The first and last two are analogous with mammalian C1, C8 and C9 respectively (Jensen *et al.*, 1973, 1981; Hyder Smith and Jensen, 1986). Bony fishes possess both CCP and ACP which can be directly compared with those of mammals. Both complement pathways have been studied in many fishes such as Japanese eel (Iida and Wakabayashi, 1983; Kusuda and Fujunaga, 1987), carp (Yano *et al.*, 1985), salmonid fishes (Nonaka *et al.*, 1981a; Ingram, 1987; Røed *et al.* 1992) and tilapia, *O. niloticus* (Matsuyama *et al.*, 1988; Yano *et al.*, 1988). Cushing (1945) observed that the first four components of the complement system in carp serum have a marked similarity to those of guinea pig complement system. Nokaka *et al.* (1981a) reported that the complement system of rainbow trout also has structural and functional similarities to the mammalian system.

A strong bactericidal activity has been reported for the complement system of many fish species. It is the ACP which is mainly activated in fish rather than the CCP during the process of bacterial killing (Koppenheffer, 1987). Components of the complement system are known to opsonise bacteria and by doing so facilitate phagocytosis of the bacteria by macrophages. Generally, fish complement exhibits an opsonic activity against non-pathogenic bacteria, but not against virulent strains of bacteria (Kusuda and Tanaka, 1988; Iida and Wakabayashi, 1993). It has been reported that C3 is the major phagocytosis-promoting factor and C3 receptors are present on the macrophages of salmonid fishes (Johnson and Smith, 1984; Sakai, 1984), tilapia (Saggers and Gould, 1989), and on the neutrophils of carp (Matsuyma *et al.*, 1992).

4.1.2.3 C-reactive protein (CRP)

C-reactive protein is found in the plasma of humans, and other mammals suffering from tissue damage, infection, or inflamation. This protein is known as CRP because of its ability to recognize and precipitate C-polysaccharide, a component of the cell wall of *Streptococcus pneumoniae*, in the presence of calcium ions. CRP is not an antibody but its amino acid composition shows some homology with that of immunoglobulins (Marchalonis and Weltman, 1971).

It has been reported that CRP is synthesized in the liver (Kushner and Feldmann, 1978). Its concentration in normal serum is around 3-5 $\mu g\ ml^{-1}$ for humans, 1.5 $\mu g\ ml^{-1}$ for rabits, 400-500 $\mu g\ ml^{-1}$ for rats, and 60 $\mu g\ ml^{-1}$ for dogs. CRP has been isolated from many species of fish including rainbow trout (Winkelhake and Chang, 1982; Murai, *et. al.*, 1990), lumpsucker, *Cyclopterus lumpus* (White *et al.*, 1978), tilapia, *O. mossambicus* (Ramos and Smith, 1978). In lumpsucker, CRP has been found in various tissues including the serum, eggs and sperms. The highest concentration of CRP was recorded in the eggs suggesting that most of the CRP synthesised in the female accumulated in the eggs (Fletcher *et al.*, 1977). In tilapia, *Oreochromis mossambicus*, a very small amount of CRP was detected in the skin and no CRP was found in the serum of normal healthy individuals. However, following physical injury, it was possible to detect CRP in the serum of the animal (Ramos and Smith, 1978).

4.1.2.4 Transferrin

Transferrins are globular, iron binding glycoproteins which are detected in the sera of vertebrates, in egg white and in mammalian milk. Each molecule of transferrin can bind

two ferric ions and thus it plays a central role in the transport of iron between sites of absorption, storage and utilisation in all vertebrates (Putnam, 1975). Iron is normally an essential element required by most infecting pathogens (Sussman, 1974). Transferrin reduces the amount of free endogenous iron by chelating it, thus making it unavailable for pathogens (Weinberg, 1974). Therefore, the amount of transferrin in the host's blood can be used as an indicator for determining the host's susceptibility to a particular pathogen.

Transferrin has been isolated from many species of fish and exhibits a high degree of genetic polymorphism between different fish types. More than 20 detectable variants have been found for human transferrin. In coho salmon, a differential resistance to bacterial kidney disease (BKD) has been found with different genotypes of transferrin (Suzumoto *et al.*, 1977). The genotype CC shows high resistance to BKD, whereas the genotypes AA and AC show high and intermediate susceptibility respectively. Røed *et al.* (1995) produced polyclonal and monoclonal antibodies against salmon transferrin and used these in an enzyme-linked immunosorbent assay (ELISA). This assay showed high sensitivity for transferrin with the lowest detectable concentration being 0.5-1.0 ng ml^{-1}.

4.1.2.5 Interferon

Interferons (IFN) are proteins or glycoproteins that can inhibit virus replication. Three types of interferons, α, β and γ have been identified in mammals. The production of IFN has also been identified in bony fishes, and Gravell and Malsberger (1965) were the first to demonstrate the production of IFN from cultured cells derived from fathead minnows. It is now known that fish produce more than one type of interferon, depending on the type of inducer and the type of cell stimulated (Alexander and Ingram, 1992). IFN is

produced mainly by sensitised lymphocytes, but also by other leucocytes. Graham and Secombes (1988, 1990) showed that leucocytes isolated from rainbow trout kidney secreted IFN-γ like molecules with antiviral and macrophage activating factor (MAF) activities when stimulated with a mitogen. Rogel-Gaillard *et al.* (1993) observed that interferon like activity was induced *in vitro* from blood and kidney leucocytes of rainbow trout by infectious or inactivated Etgved virus, viral hemorrhagic septicemia virus (VHSV) serotypes.

There are also other substances involved in the non-specific defence system of fish against pathogens. These include lectins (Goldstein *et al.*, 1980), natural agglutins (Sindermann and Honey, 1964), precipitins (Janssen and Meyers, 1968), naturally-occurrring immunoglobulins (Leslie and Clem, 1970) and non-immunoglobulin antibody-like molecules (Marchelonis and Weltman, 1971).

4.1.3 Non-specific immune system : cellular defence

Fish possess a variety of non-specific leucocyte types including monocytes, macrophages, granulocytes, and non-specific cytotoxic cells (NCCs). They include macrophages and granulocytes which are mobile phagocytic cells found in the blood and secondary lymphoid tissues. These cells are particularly important in inflammation resulting from microbial invasion or tissue damage and respond by migrating to the site of inflammation where they kill the pathogens. Although the cellular activities they elicit are non-specific in nature, they are able to interact with cells of the specific immune system, thus stimulating these cells and inducing a specific response.

4.1.3.1 Macrophages

Macrophages are normally found in blood, lymphoid organs such as kidney, spleen, and in the peritoneal cavity. They are mononucleated, derived from circulating monocytes have an undulating membrane and have characteristically high levels of phagocytic or pinocytic activity (Laskin and Lechevalier, 1972). The size of macrophages is variable and depends on the species of fish examined, and the state of digestion of ingested material (Ellis, 1976; Russell, 1974). During differentiation of a monocyte to a macrophage, the cell enlarges five to ten fold and its intracellular organelles increase in both number and complexity, and acquires increased phagocytic activity by producing higher levels of lytic enzymes. Their surface becomes irregular and develops several finger-like pseudopodia which extend during phagocytosis. The large nucleus of the macrophage is fairly irregular in outline with a slightly marginated nuclear chromatin (Timur, 1975).

Macrophages are normally dispersed throughout the body. Some of them reside in particular tissues where they have become fixed macrophages, whereas others remain motile and are called free, or wandering macrophages. The free macrophages move by amoeboid movement throughout the tissues. On the other hand, fixed macrophages serve different functions depending on the tissues where they reside. In fish, the spleen is composed of highly phagocytic macrophages, within a reticulin fibre network, called an ellipsoid, in which immune complexes are trapped (Ellis, 1982). The macrophages normally in a resting state, can be activated by a variety of stimuli in the course of an immune response. Phagocytosis of particulate antigens serves as an initial activating stimulus. Macrophage activity can be further enhanced by cytokines secreted by activated T_H cells, by mediators of the inflammatory response, and by bacterial cell-wall products.

One of the most potent activators of macrophages is interferon-γ secreted by activated T_H cells. Ruco and Meltzer (1978) reported that macrophages were activated in two ways. Firstly, inactivated macrophages were primed by the stimulus of low-dose lipopolysaccharide (LPS), thioglycolate, complement, lymphokine or interferon-γ. In the primed state, macrophages showed increased spreading, alteration in the cell surface receptors and increased metabolic activity. Unactivated macrophages can be fully activated *in vitro* by either phorbol myristate (PMA), mitogens including concanavalian A (Con A) and LPS or by calcium ionophore A23187 (West, 1990). Compared with unactivated or resting macrophages, activated macrophages are more efficient in eliminating potential pathogens because they exhibit greater phagocytic activity, increased secretion of inflammatory mediators, and an increased ability to activate T cells. Activated macrophages produce a number of reactive oxygen and nitrogen intermediates that have potent antimicrobial activity. During phagocytosis, a metabolic process known as respiratory burst occurs in activated macrophages. This process results in the activation of a membrane-bound oxidase (NADPH) that catalyses the reduction of oxygen to superoxide anion, a reactive oxygen intermediate which is extremely toxic to ingested microorganisms. The superoxide anion also generates other powerful oxidising agents, including hydroxyl radicals, singlet oxygen, and hydrogen peroxide. Macrophages have large phagosomes where they digest all sorts of foreign substances, necrotic debris and even red blood cells. Where possible they convert them into a soluble form so that they can be utilized by the body, eliminated as waste or used to prime the immune system to stimulate an immune response (Ellis, 1976, 1977, 1981; Phromsuthirak, 1977).

Functionally, macrophages act as a surveillance system for the body. They also act as accessory cells for the lymphocyte response. Activated macrophages express higher levels of class II MHC molecules in the cell surface, allowing them to function more effectively as antigen-presenting cells (Kuby, 1997).

4.1.3.2 Granulocytes

Granulocytes are generally the first cell to respond in inflammation and destroy the invading organisms by phagocytosis or cytotoxic killing (Finn and Nielson, 1971; MacArther *et al.*, 1984). In fish, granulocytes are composed of neutrophils, eosinophils, basophils and mast cells, but the relative proportion of the different populations vary from one species to another (Rowley *et al.*, 1988).

Granulocytes can be isolated along with macrophages from blood, lymphoid tissues, and the peritoneal cavity, and they can be further isolated from the macrophages. Lamas and Ellis (1994) observed that granulocytes adhere to culture vessels, especially if they are pre-coated with celloidine. The most conspicuous feature of isolated granulocytes are the granules present in their cytoplasm. The granules can be stained with dyes like Sudan black or enzymes such as peroxidase, and can be used to identify the cell type. Granulocytes are polymorphonuclear and this characteristic can also help to identify them. Isolated granulocytes, specially neutrophils, are highly mobile, phagocytic and produce reactive oxygen species, but their bactericidal activity is often relatively poor compared to macrophages.

4.1.3.3 Phagocytosis

Phagocytosis is a process in which phagocytic cells internalize, kill and digest invading microorganisms. Macrophages and granulocytes are the most active and directly involved phagocytic cells. *In vivo* and *in vitro* studies on phagocytosis demonstrate that monocytes/macrophages and granulocytes (mostly neutrophils and in some cases eosinophils) are phagocytic and can ingest a wide range of inert and antigenic particles (Ainsworth, 1992; Secombes and Fletcher, 1992; Steinhagen and Jendrysek, 1994), and soluble ligands (Dannevig *et al.*, 1994). Macrophages are capable of ingesting and digesting exogenous antigens such as whole microorganisms, insoluble particles, injured and dead host cells, cellular debris, and activated clotting factors. Thrombocytes are also described as phagocytic, but their phagocytic activity is very low and their capability for intracellular digestion still remains unclear.

Phagocytosis normally occurs in three steps : i) attachment of the particle to the cell membrane, ii) ingestion involving the formation of a phagosome, and iii) breakdown of the particle within the phagosome. In the first step of phagocytosis, adherence of the antigen to the phagocyte membrane is essential and a passive process. Phagocytes are attracted by and move toward the antigen, as a result of stimuli from the immune response, a process known as chemotaxis. Fish phagocytes have the capacity of distinguishing between targets, suggesting the involvement of surface receptors. Phagocytosis can take place *in vitro* in the absence of serum, indicating the presence of a number of lectin-like receptors on macrophages. It has been shown with tilapia (*O. spilurus*) macrophages, pre-incubation of macrophages with either L-frucose, D-galactose, D-glucose, D-mannose, α-methyl mannosile, or N-acetyl-D-glucosamine significantly inhibits phagocytosis (Saggers and

Gould, 1989). As the macrophage membrane possesses receptors for certain classes of antibody and certain complement components, its adherence to the antigen can be increased by coating the antigen with the appropriate antibody or complement components. This process is known as opsonization. It has been observed that opsonization of particles with hemolytically active normal serum greatly increases their adherence to macrophages and neutrophils, and subsequent ingestion (Matsuyama *et al.* 1992; Rose and Levine, 1992). Johnson and Smith (1984) successfully used mammalian complement components to opsonize particles. Serum component, CRP, has also been demonstrated to act as an opsonin in fish (Nakanishi *et al.*, 1991).

Ingestion of an antigen is an active process and can occur by engulfment or enfoldment. During engulfment, the macrophages extend their pseudopodia around the attached antigen and fuse with it. Fusion of the pseudopodia encloses the antigen within a membrane-bound structure called a phagosome, which then enters the endocytic processing pathway. In this pathway, a phagosome moves toward the cell interior, where it fuses with a lysosome to form a phagolysosome. The lysosome contains hydrogen peroxide, oxygen free radicals, peroxidase, lysozyme, and various hydrolytic enzymes which digest the ingested material.

4.1.4 Characteristics of *Aeromonas hydrophila* and its infection

A. hydrophila is a bacteria and is a suitable fish pathogen for artificially producing disease in tilapia. Therefore, *A. hydrophila* was used as the challenge strain in testing disease susceptibility between the clonal lines in this study.

The genus *Aeromonas* is naturally divided into two groups, motile and non-motile. The mesophilic, motile group comprises *A. hydrophila* (synonym, *A. liquefaciens*), *A. sorbia*, and *A. caviae* (synonym, *A. hydrophila* subsp. *anaerogenes*). All three species of motile aeromonads have been isolated from fish, although *A. hydrophila* is the most frequently isolated and most significant pathogen of the three (Frerichs and Millar, 1993).

A. hydrophila has been grouped under the family Vibrionaceae. It is a gram-negative, rod shaped, motile microorganism, normally living in soil and water, and is capable of infecting cold-blooded vertebrates and mammals (Bullock *et al.* 1971; Popoff and Veron, 1976; Ho *et al.* 1990). It is distributed widely in clean and organically polluted freshwater, water with high sewage levels, and also in the marine environment except where the water salinity is very extreme (Hazen *et al.*, 1978; Heuschmann-Brunner, 1978; Kaper *et al.*, 1981; Newman, 1982). *A. hydrophila* has been identified as a constituent of intestinal micro flora of healthy fresh water or sea water fishes (Thorpe and Roberts,1972; Sakata *et al.*, 1980; Newman, 1982). It has also been identified as primary and secondary pathogens in a number of aquatic and terrestrial animals including humans (Howard and Buckley, 1985).

A. hydrophila are characterised by active motility from a single polar flagellum, and production of gas and acid from carbohydrates. Morphologically, they are straight rods measuring 0.5 x 1.0-1.5 µm, facultatively anaerobic, non-spore forming, and resistant to the vibriostat 0/129. They are cytochrome oxidase positive and are able to reduce nitrates. The colonies of *A. hydrophila* are generally white to buff, circular, smooth and convex, and can be formed within 24 hrs at 22-28 °C. The optimum temperature for *A. hydrophila* culture is 25-30 °C, but they can grow at a range of temperatures between 0-45 °C. They are readily

isolated on any general purpose medium, such as tryptone soy agar, brain heart infusion agar and sheep blood agar (Bullock, 1961; Rouf and Rigney, 1971; Newman, 1982; Frerichs and Roberts, 1989; Roberts, 1993). *A. hydrophila* can be differentiated from the other two motile aeromonads (*A. sorbia* and *A. caviae*) on the basis of carbohydrate and other biochemical reactions. However, they can be characterized by a variety of properties defined by Popoff and Veron (1976) and Popoff (1984).

Association of aeromonads with disease was first recognised when Sanarelli (1891, cited by Roberts, 1993) reported an outbreak of a disease in eels associated with what is presumed to have been *A. hydrophila* or *Vibrio anguillarum*. Apart from fish, *A. hydrophila* has also been isolated from diseased frogs, alligators, turtles, shrimps, man (Newman, 1982) and snails (Mead, 1969). The main feature of the pathogenesis of all *A. hydrophila* infections in fishes is a generalized dissemination in the form of bacteraemia, followed by elaboration of toxins, tissue necrosis and the clinical disease known as haemorrhagic septicaemia. Bacterial haemorrhagic septicaemia results from the infection of *A. hydrophila* possibly transmitted to other animals through water, via diseased and healthy carrier fish, and other affected invertebrates. The disease is also associated with external and internal parasites (Newman, 1982). Frerichs (1989) reported that *A. hydrophila* is considered to be the principal cause of bacterial haemorrhagic septicaemia in fresh water fish, and is associated with a variety of ulcerative conditions including epizootic ulcerative syndrome (EUS) in South-East Asia (Llobrera and Gacutan, 1987; Lio-Po *et al.* 1992; Millar, 1994). Stress factors, such as crowding, excessive handling, high water temperature, low dissolved oxygen levels, malnutrition and also seasonal fluctuations are all possible causes of *A. hydrophila* infection to fish (Rock and Nelson, 1965; Haley *et al.*, 1967; Shotts *et al.*, 1972;

Snieszko, 1974). Due to the ubiquitous distribution of the organism, fish can be at risk at any time (Frerichs and Roberts, 1989). Snieszko and Axelord (1971) classified disease caused by *A. hydrophila* into the four following categories i) acute, rapidly fatal septicaemia with few clinical signs, ii) an acute form with dropsy, blisters, abscesses and scale protrusion, iii) a chronic ulcerative form with furuncles and abscesses, and iv) a latent form with no signs of infection.

In this bacterial challenge, clonal lines of tilapia were used to examine the genetic variation in disease resistance between the lines. The clonal lines are homozygous for every gene locus and each line may differ for a particular allele for genes involved in non-specific immune response which is expected to show some variations in disease resistance. Heterozygous clones were also produced which when compared to their parental clones may suggest genetic basis of any response.

In the above sections, a general background of the non-specific immune system of fish has been discussed. But in this study, some immunological parameters such as serum lysozyme activity, phagocytosis and respiratory burst of head kidney macrophages, haematocrit, total number of white and red blood cell counts, differential white blood cell counts and susceptibility of clonal lines to an artificial bacterial challenge have been studied.

4.2 Materials and methods

4.2.1 Experimental fish

The non-specific immune response of clonal lines of *O. niloticus* was examined here. The inbred clones were produced from mitotic gynogenetic females using gynogenesis as previously described in Chapter 2. A few groups of gynogenetic outbred or heterozygous clones were also produced by crossing two different clonal lines again explained in Chapter 2. The unrelated control fish were produced by ordinary crossing. All of the experimental fishes were reared and maintained in the recirculating water system described in Section 2.2.1.1.

4.2.2 Lysozyme assay

Lysozyme activity was measured according to Parry *et al.* (1965) using a turbidity asssay in which 0.2 mg ml^{-1} lyophilized *Micrococus lysodeikticus* in 0.04 M sodium phosphate buffer, pH 5.75 (Appendix 4.1) was used as substrate. Forty µl of fish serum was added to 3 ml of the bacterial suspension and the reduction in absorbance at 540 nm determined after 0.5 min and 4.5 mins incubation at 22 ^{o}C. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 per min.

4.2.3 Phagocytic assay

4.2.3.1 Macrophage isolation and monolayer preparation

Macrophages were isolated from head kidney of tilapia, *O. niloticus* by following the methods described by Secombes (1990). Head kidney tissue was collected from fish using sterile forceps and scalpel blades. The collected kidney was weighed in a sterile vial

with L-15 medium before isolation of macrophages to determine the total number of macrophages per g of tissue. The isolated kidney was placed in a sterile Petri dish containing 5 ml of Leibovitz-15 (L-15) medium (Sigma) and 20 i.u. ml^{-1} heparin. The tissue was teased through a sterile 100 µm nylon mesh to form a cell suspension. This procedure was performed on ice. Cell suspensions were carefully placed onto a previously prepared 34%/51% (v/v) Percoll gradient. Macrophages were separated from the cell suspension by centrifuging the gradient at 400 g for 25 mins at 4 °C. The band at the interface of the 34%/51% fractions, enriched with macrophages, was collected with a sterile pipette. The cells were washed twice with fresh L-15 medium by centrifuging at 1000 g for 7 mins at 4 °C. The viable cells were then counted on a haemacytometer using trypan blue. All cells in the four large squares of the haemacytometer were counted and their number calculated using the following formula:

Cells ml^{-1} = average cell number per large square x 1/dilution x 10^4

The concentration of macrophages was adjusted to $5x10^6$ ml^{-1} using L-15 medium containing 1% penicillin/streptomycin (pen/strep).

Macrophage monolayers were prepared by placing 300 µl aliqouts of the cell suspension onto circles of a sterile frosted glass slide (Dynex Laboratories: 3 wells per slide, 14 mm diameter per well) and incubated at 22 °C for 3 hrs in a humid chamber. Following incubation, non-adhering cells were removed by washing three times with L-15 medium. It was important to use medium of the same temperature as the incubated cells (22

°C) since cold medium facilitates the detachment of adherent cells. After washing, 300 µl of L-15 medium containing 5% foetal calf serum (FCS) and 1% pen/strep was added to each well. The slides were then placed in a humid chamber and incubated at 15 °C for 24 hrs.

4.2.3.2 Determination of adherent macrophage numbers

The actual number of adherent macrophages in the monolayers was counted using a haemacytometer. The monolayer was washed once with L-15 medium, then 300 µl of lysis buffer (Appendix 4.2) was added to each monolayer and mixed well by pipette. After 2 mins, the cell suspension was applied to the haemacytometer, left for 2 mins to allow cells to settle and nuclei released from the lysed cells to be counted. The nuclei of macrophages were counted following the methods described in Section 4.2.3.1.

The determination of macrophage numbers is important so as to be able to adjust the bacteria : macrophage ratio for phagocytosis.

4.2.3.3 Engulfment of bacteria by macrophages

Head kidney macrophage monolayers were prepared for phagocytosis and washed once with L-15 medium before use. A 300 µl aliquot of *Aeromonas salmonicida* (strain MT 004) suspension ($5x10^7$ bacteria ml^{-1}) was added to each monolayer at a ratio of one macrophage to ten bacteria.

Before adding bacteria to the monolayers, they were opsonized with 20% (v/v) fresh serum from naive fish. Phagocytosis of *A. salmonicida* was carried out for 1 h at 22 °C, after which the macrophages were washed 5 times with phosphate buffer saline (PBS), pH 7.2 (Appendix 4.3), fixed and stained with a Rapi Diff II staining system (Raymond A

Lamb-Laboratory Supplies, London, UK). The slides were mounted and examined under oil by light microscopy. Macrophages, able or unable to phagocytose bacteria, were determined. Control macrophages without bacteria were also prepared for comparisons.

4.2.3.4 Preparation of bacteria, *Aeromonas salmonicida*

A. salmonicida (strain MT 004) was used for phagocytosis. Bacteria were cultured on tryptone soy agar (TSA) (Appendix 4.4.1) plates at 22 °C and maintained by subculturing plates every 4 to 5 days. One or two colonies were taken from the TSA plate and seeded into tryptone soya broth (TSB) (Appendix 4.4.2) overnight at 22 °C. The culture was washed twice with sterile PBS by centrifuging at 800 g for 10 mins at 4 °C in the following morning. The resultant pellet was then resuspended in sterile PBS and their concentration determined spectrophotometrically at 610 nm, according to Thompson (1993).

4.2.4 Respiratory burst

A measurement of respiratory burst of head kidney macrophages from tilapia was carried out in 96 well microtitre plates according to Secombes (1990). Aliquots of 100 µl macrophage cell suspension were placed in each well and macrophage monolayers were prepared following the procedure explained in Section 4.2.3.1. Before adding NBT or PMA to the wells, the macrophage monolayers were washed with L-15 medium and the number of adherent cells in each monolayer determined using lysis buffer following the methods described in Section 4.2.3.2. A 100 µl aliquot of NBT, dissolved at 1 mg ml^{-1} in L-15 medium was added to half of the wells, and NBT, at the same concentration, with added

PMA (1 µl ml^{-1} of L-15 medium) was placed in the other half of the wells. The plate was incubated for 1 h at 20 °C. The medium was removed from the wells and the cells were fixed with 100% methanol for 5 mins. The cells were washed several times with 70% methanol and allowed to air dry. An aliquot of 120 µl of 2M KOH and 140 µl of dimethyl sulfoxide was added to each well to dissolve the blue formazan generated by the reduction of NBT. The turquoise-blue coloured solution in the wells was then read in a multiscan spectrophotometer at 610 nm.

4.2.5 Counting of white and red blood cells

Blood was collected from the caudal vein of anesthetized tilapia by using 21 g sterile needles. Both erythrocytes and leucocytes were counted using a haemacytometer. The blood was diluted 1/1000 in PBS to count erythrocytes whereas blood was diluted 1/100 in PBS for leucocytes. Differential white blood cell counts were carried out by staining blood smears with Rapi Diff II staining system.

4.2.6 Haematocrit measurements

Heparin coated haematocrit capillary tubes were filled with blood and one end sealed with Cristaseal. The tubes were later centrifuged at 10,000 rpm for 3 mins in a haematocrit centrifuge (Hawksley and Sons Ltd., Lancing, W. Sussex). The haematocrit was expressed as a percentage of packed red blood cells against total blood volume.

4.2.7 Bacterial challenge

4.2.7.1 Preparation of inoculum

Susceptibility to a bacterial challenge *in vivo* was carried out with different clonal lines of tilapia, *O. niloticus*. *A. hydrophila* (strain T4) isolated from *L. rohita* during an outbreak of epizootic ulcerative syndrome in Bangladesh (Millar, 1994) was used as the challenge strain. The bacteria was cultured in TSB for 24 hrs at 22 °C following removal from -70 °C preservation. The broth culture was plated out onto TSA for 24 hrs at 22 °C to check the purity of the culture and this was used as a stock plate. One or two colonies of the bacteria were removed from the plate and cultured in TSB medium for 24 hrs at 22 °C. The bacteria were harvested by centrifuging at 800 g for 10 mins. The resultant pellet was washed twice with sterile PBS and then finally resuspended in PBS. Bacterial concentration was determined by relating the absorbance of the suspension at 610 nm to a pre-made standard curve relating concentration to absorbance. This curve is presented in Appendix 4 Figure 1. The bacterial suspension was adjusted to $5x10^6$ cells ml^{-1} with sterile PBS and used for the challenge.

4.2.7.2 Standardisation of optimum dose of *A. hydrophila* (T4) for challenge

A preliminary experiment was performed to optimise the dose of *A. hydrophila* for challenge.

Initially three fish were used. A bacterial suspension was prepared following the procedure described above. Suspensions of the bacteria were prepared at concentrations of $1x10^8$, $1x10^7$ and $1x10^6$ cells ml^{-1}. Viable plate counts of bacterial suspensions were also prepared for each and the exact concentrations used were $1.6x10^8$, $1.5x10^7$ and $1.5x10^6$ cells

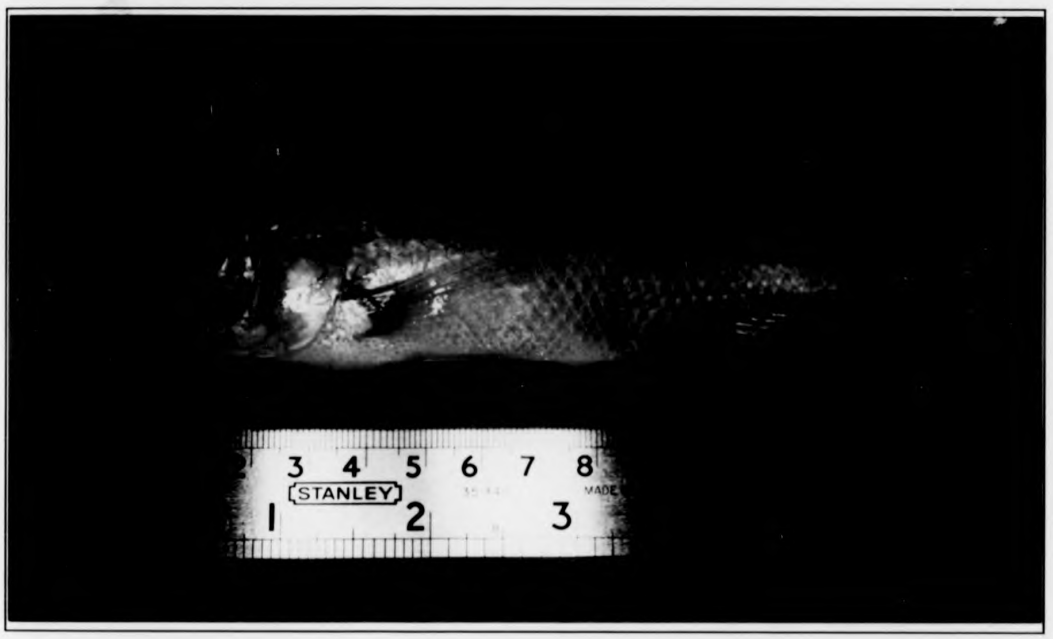
ml^{-1}respectively. A dose of 0.1 ml of each of the three suspensions were injected intraperitoneally (ip) into three fish (one fish for each concentration). All three fishes died within 12-20 hrs after injection. Symptoms of infected fish included slow movement and remaining near the surface of the water. Examination of the gross pathology of dead fish showed a marked hyperemia and petechial haemorrhage on the body surface, base of fins and external site of the operculum. The anus was reddish, the abdomen was swollen, and the injection site was sometimes swollen and haemorrhagic (Figure 4.1).

Since the first bacteria challenge was too severe it was repeated with a lower dose of bacteria. Five fish and five different bacterial concentrations ($1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$ and $1x10^3$ cells ml^{-1}) were used. The viable counts of the suspensions were $0.9x10^7$, $0.9x10^6$, $0.8x10^5$, $1.1x10^4$, and $0.9x10^3$ bacteria ml^{-1} respectively. The fish which were injected i.p. with 0.1 ml of $1x10^7$ bacteria ml^{-1} died within 16 hrs. The dead fish all showed typical gross pathology of *A. hydrophila* infection as described above. Fish injected with the other concentrations did not die, and no signs of infection were evident.

The results of previous two trials showed that fish injected with $1x10^7$ cells ml^{-1} died within 16 hrs, and in the second trial no mortalities occurred in fishes injected with $1x10^6$ cells ml^{-1} or less. Therefore, it was assumed that a concentration between $1x10^7$ and $1x10^6$ cells ml^{-1} would be an ideal dose to produce sufficient clinical signs of infection, but keep the level of mortality to a minimum. Four bacterial suspensions containing $1x10^7$, $5x10^6$, $1x10^6$ and $5x10^5$ cells ml^{-1} were prepared and viable plate counts showed the bacterial concentrations to be $0.8x10^7$, $5.3x10^6$, $1.2x10^6$, $5.5x10^5$ cells ml^{-1} respectively. Each suspension was injected i.p. into four fish with a dose of 0.1 ml per fish. All fish injected with $1x10^7$ bacteria ml^{-1} died within 20 hrs post injection, two fish injected with

Figure 4.1 *O. niloticus* injected with *Aeromonas hydrophila* (strain T4) showing haemorrhage on the body surface, base of fins and the operculum

ORIGINAL IN COLOUR


3 4 5 6 7 8
STANLEY
1 2 3

$5x10^6$ bacteria ml^{-1} died within 30 hrs and another two fish from this group survived but had internal and external signs of infection. Fishes injected with other concentrations did not die and no bacteria were re-isolated from these fish. Therefore, from the above results, 0.1 ml of $5x10^6$ bacteria ml^{-1} was used as the dose for the challenge experiments.

4.2.8 Statistical analysis

The data from the haematocrit measurements, serum lysozyme activities, differential WBC counts and respiratory burst obtained for the different groups of fish were analysed by one way ANOVA using a significant level of $P<0.05$. Significant results were further analysed by Tukey's pairwise comparison test to identify significant differences between means.

In order to determine the level of phagocytic activity, a total of two hundred macrophages were counted per fish and phagocytosis was classified as cells which had phagocytosed 0, 1-9 or ≥10 bacteria. Interspecific comparisons for each macrophage classification were made by Kruskal-Wallis non-parametric analysis of variance.

Results of the bacterial challenge (infected and non-infected) were analysed by Fisher's Exact Test with a significant level of $P<0.05$.

4.3 Design of the experiments to study the non-specific immune response of clonal lines

Two studies were performed to examine the non-specific immune responses of the clonal groups of tilapia.

4.3.1 Experiment 1

A preliminary study was carried out with a small number of fish to standardize the immunological parameters for tilapia.

4.3.1.1 Immunological assays

An array of immunological assays were performed according to Thompson *et al.* (1996) to compare non-specific immune responses between groups of clonal fish. The assays tested included haematocrits, differential white blood cell (WBC) counts, serum lysozyme, and head kidney macrophage activities including phagocytosis and respiratory burst. Three groups of fish, clone 002 041 887, its related control and an unrelated control were used to carry out preliminary studies. For convenience, the clonal line was designated by the mitotic mother's tag number (002 041 887). A total of 15 fish from each group were used. One fish per group was sampled on each sampling day and the assays described above performed.

4.3.1.2 Bacterial challenge

Bacterial challenge was carried out on the different groups of tilapia to examine their susceptibility to *A. hydrophila* infection. Three groups of fish, inbred clone 009356316 (ICL A), inbred clone 010036092 (ICL B) and an unrelated control group (URC), 12 fish from each group were used in the challenge.

At least two weeks before the challenge, fish (50-56 g) were transferred to the challenge room to allow acclimatisation to their new environment. They were maintained in six 120 l aquaria divided into three sections with Perspex partitions. Four fish from each group were placed in each section to give a total of 12 fish per tank. Fish in three of the tanks were injected i.p. with 0.1 ml of the bacterial suspension ($5x10^6$ cells ml^{-1}). The same volume of sterile PBS was injected into fish in the three remaining tanks. These were used as control fish. The challenge experiment was continued for a period of six days after injection and during this period no food was given to any of the fish. To maintain the water quality, 2/3 of the aquarium water along with solid wastes was siphoned out twice a day using a disinfected PVC pipe and the aquarium was then filled up with fresh warmed water (28 $\pm 1^{o}$C). Additional aeration was also provided to each aquarium. After injection, fish were observed at 4 h intervals for mortalities. All dead fishes were removed and bacterial swabs taken from the outer edges of skin lesions and from their kidney, and cultured on TSA plates. Isolated *A. hydrophila* was confirmed using conventional methods (Cowan and Steel, 1993) and the API 20E system (bio Mericux, France) (Appendix 4.5).

4.3.1.3 Results

4.3.1.3.1 Immunological assays

The non-specific immune responses determined included serum lysozyme activity, haematocrit values, differential white blood cell counts and phagocytosis and respiratory burst of head kidney macrophages between the different groups of fish. The haematocrit, differential WBC counts and respiratory burst results did not show any significant difference between the three groups of fish (Table 4.1). The lysozyme activity in the serum of the clonal line, related and unrelated controls were significantly different ($P<0.05$) from each other (Figure 4.2). During phagocytosis, macrophages of fish extend their

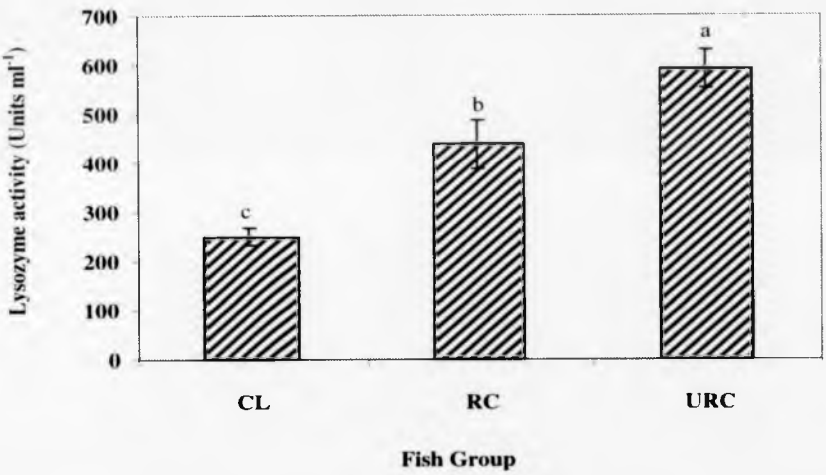


Figure 4.2 Serum lysozyme activity in the different groups of tilapia, *O. niloticus*.

CL-Clone 002041887, RC-Related control, URC-Unrelated control

Column with different letters (a, b, c) indicate significant difference at $P<0.05$
Fifteen fish were sampled per group

Table 4.1 Lysozyme activity, haematocrit measurement, and respiratory burst and phagocytosis of head kidney macrophages in different groups of tilapia, *O. niloticus*

Fish group	Serum lysozyme activity (Unit ml^{-1}) (n=15)*	Haematocrit (%) (n=13)	Respiratory burst (n=14)[p]		No. of macrophages contain phagocytosed bacteria (%) (n=5)[q]		
			NBT	PMA	0 bacteria	1-9 bacteria	≥ 10 bacteria*
Clone 002041887	249.54 ± 18.78[c]	37.65 ± 1.0	0.015 ± 0.005	0.041 ± 0.008	59.64 ± 3.78	36.38 ± 3.64	3.97 ± 1.03[b]
Related control	437.69 ± 49.30[b]	38.40 ± 0.60	0.010 ± 0.003	0.028 ± 0.004	65.63 ± 1.84	29.09 ± 2.91	5.28 ± 0.97[b]
Unrelated control	590.36 ± 39.54[a]	36.06 ± 0.85	0.011 ± 0.002	0.051 ± 0.009	54.77 ± 3.88	34.41 ± 3.71	10.82 ± 1.46[a]

[p] expressed as a mean absorbance of at least twelve replicate wells per fish at 540 nm for $1x10^5$ cells per well. NBT- nitroblue tetrazolium, PMA- phorbol myristate acetate

[q] 200 macrophages were counted per fish

Numbers with different letters within a single column (a, b, c) indicate significant difference at $P<0.05$

* indicate significant correlation ($P<0.05$) between serum lysozyme activity and phagocytosis of macrophages conaining ≥ 10 bacteria

pseudopodia around the bacteria and engulf them (Figure 4.3). The percentage of macrophages able to phagocytose bacteria from the three groups of fish is presented in Figure 4.4. Fish in the three groups contained similar numbers of macrophages which were unable to phagocytose bacteria or were able to phagocytose between 1 and 9 bacteria, but the number of macrophages containing $\geq$10 bacteria was significantly higher ($P<0.05$) in the unrelated control group compared with the other two groups.

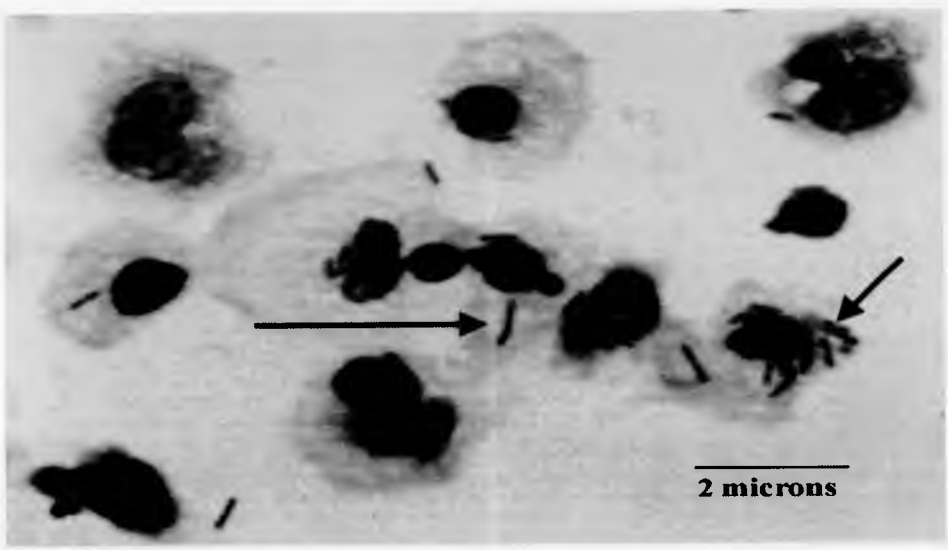


Figure 4.3 Phagocytosis of bacteria by head kidney macrophages in tilapia, *O. niloticus*
Arrows showing the phagocytosed bacteria in the cytoplasm of macrophages

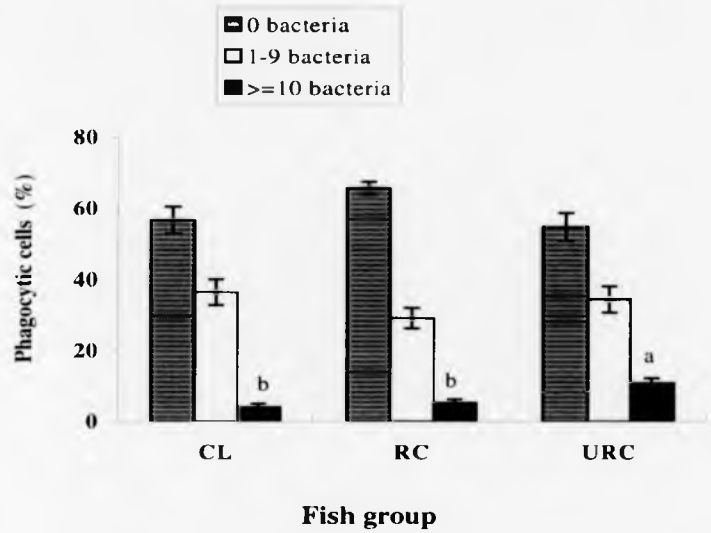


Figure 4.4 Phagocytic activity of macrophages in different groups of tilapia, *O. niloticus*

CL- Clone 002 041 887, RC-Related control, URC-Unrelated control

Column with different letters (a, b) indicate significant difference at $P<0.05$
Five fish were sampled per group and 200 macrophages were counted per fish

4.3.1.3.2 Bacterial challenge

After i.p. injection with *A. hydrophila*, fish in all groups showed slow movement and stayed near the surface of the water. They started to die 12 hours after injection. The gross pathology of the dead fish showed a typical *A. hydrophila* infection as described in Section 4.2.7.2. Fish mortalities due to *A. hydrophila* infection occurred over the course of the first two days of the challenge with 50 % mortalities in ICL B, 16.7 % in ICL A and 41.67 % in the URC. The cumulative mortalities of different groups of fish during

bacterial challenge are presented in Figure 4.5. Six days after injection, fish which had survived were sampled for *A. hydrophila*, but it was only possible to re-isolate the bacteria from ICL B fish with 33.3 % of the surviving fish carrying the bacterium (Figure 4.6). Table 4.2 shows the response of different groups of tilapia to *A. hydrophila* infection. The analysis of total infection (mortality plus survived) in different groups of fish showed that ICL B had a significantly higher ($P<0.05$) number of infected fish than ICL A and the URC group. No deaths or infection were found in the control group injected with sterile PBS.

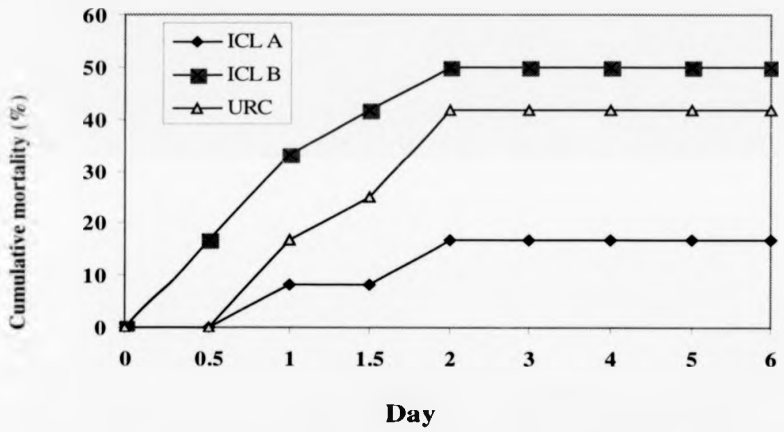


Figure 4.5 Cumulative mortalities (%) of tilapia during an artificial challenge by *A. hydrophila* (T4)

ICL A-Inbred clone 009356316, ICL B-Inbred clone 010036092
URC-Unrelated control

Twelve fish were used per group

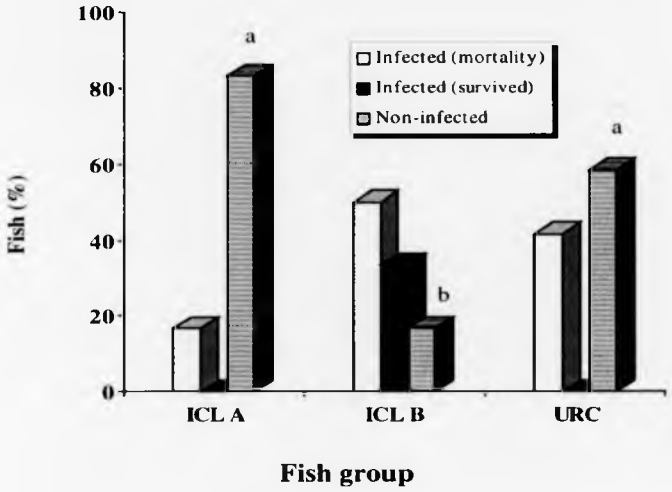


Figure 4.6 Susceptibility of tilapia to *A. hydrophila* after an artificial challenge

ICL A- Inbred clone 009356316, ICL B- Inbred clone 010036092, URC-Unrelated Control

Column with different letters (a, b) indicate significant difference at $P<0.05$

Twelve fish were used per group

Significant infection levels in different fish groups are also presented in Table 4.2

Table 4.2 Response of different groups of tilapia, *O. niloticus* to an artificial bacterial challenge with *A. hydrophila*

Fish group	No of fish injected	Infected (mortality)	Infected (survived)	Non-infected
ICL A[a]	12	2	0	10
ICL B[b]	12	6	4	2
URC[a]	12	5	0	7

ICL A-Inbred clone 009 356 316; ICL B-Inbred clone 010 036 092; URC-Unrelated Control

Fish groups with different superscript letters (a, b) indicate significant difference at $P<0.05$ for their levels of infection

4.3.2 Experiment 2

Due to the unavailability of sufficient clonal groups of fish, it was not possible to directly compare the non-specific immune responses obtained between clonal groups in the first experiment with fish used for the bacterial challenge. The purpose of the first experiment was mainly to standardise immunological parameters for tilapia and the results of this could then be used as base line information for the second experiment. After the first experiment sufficient inbred and outbred clonal groups of fish were produced, so as to carry out a comparative study of the non-specific immune responses between clonal groups and their resistance to a bacterial challenge.

4.3.2.1 Immunological assays

In order to compare the immune responses between inbred and outbred clonal lines, most of the immunological assays performed in the previous experiment were conducted here. Three inbred clonal lines (ICL), three outbred clonal lines (OCL) and an unrelated control (URC) group were used. The inbred clonal lines were designated as before by the mitotic mother's tag number, 009356316 (ICL A), 010036092 (ICL B), 006812566 (ICL C) and the outbred clonal lines were designated by the parents tag number, 009356316 x 010036092 (OCL AxB), 010036092 x 006812566 (OCL BxC) and 006812566 x 009356316 (OCL CxA). The outbred clones were produced by crossing a mitotic female from one line with a sex reversed male from another line (see Section 2.2.12.2). A total of 70 fish, 10 from each group were used in the analyses, and one fish from each group was sampled on each sampling day.

The data from the haematocrits, lysozyme activity and differential WBC counts and the levels of phagocytic activity of macrophages of different groups of fish were analysed following the methods described in Section 4.2.8.

4.3.2.2 Bacterial challenge

An artificial bacterial challenge with *A. hydrophila* (strain T4) was carried out using the three aforementioned inbred clonal lines, ICL A, ICL B, ICL C and three outbred clonal lines, OCL AxB, OCL BxC, OCL CxA. A total of 24 Perspex tanks each holding 30 l water were used to maintain the fish. Forty fish (average weight 62.0 g) from each group were used in the bacterial challenge, with 10 fish placed in each tank. Fish in three of the tanks for each group were subjected to bacterial injection and fish in another tank were used as control. A bacterial suspension with a concentration of 5 x 10^6 cells ml^{-1} was prepared following the procedure described in Section 4.2.7.1 and 0.1 ml of the suspension was injected i.p. into anaesthetised fish. The same volume of sterile PBS was injected into control fish. After injection, fish were transferred into the tanks and maintained as explained in Section 4.3.1.2. The fish were observed regularly at 4 hrs intervals for behavioural changes and mortalities. All dead fish were immediately removed from the tanks, bacterial swabs taken from the kidney and cultured according to Section 4.3.1.2. Levels of infection obtained for the different groups of fish were analysed as mentioned in Section 4.2.8.

4.3.2.3 Results

4.3.2.3.1 Immunological assays

Non-specific immune responses in the inbred and outbred clonal lines and an unrelated control group were examined. For this study, a variety of basic immunological parameters such as serum lysozyme activity, haematocrit measurement, differential WBC counts, RBC counts, estimate of macrophage number per g of head kidney and phagocytosis of macrophages were carried out. The lysozyme activity in the serum of ICL C was significantly higher ($P<0.05$) than in ICL A and ICL B, but the latter two groups were not significantly different to each other. The OCLs showed an intermediate level of serum lysozyme activity to that of their parents. The lysozyme activity in OCL BxC and OCL CxA were significantly higher ($P<0.05$) than the ICL A, ICL B and OCL AxB, and significantly lower than the ICL C, but they were not significantly different to each other. On the other hand, the URC group showed significantly higher ($P<0.05$) level of lysozyme activity than ICL A, ICL B and OCL AxB (Figure 4.7 and Table 4.3).

The analysis of haematocrit showed ICL A and ICL C had similar significantly low values compared to all other groups which were not significantly different from each other (Table 4.4).

In the phagocytic assay, the macrophages which phagocytosed bacteria were classified into three categories, macrophages with no phagocytosed bacteria, macrophages containing one to nine (1-9) bacteria and macrophages containing ten or more (≥10) bacteria (Table 4.3). Significant differences were observed between the different groups of fish in the number of their macrophages containing 0 and ≥10 phagocytosed bacteria, but the number of macrophages with 1-9 bacteria were almost the same between fish groups. The number of macrophages which were unable to

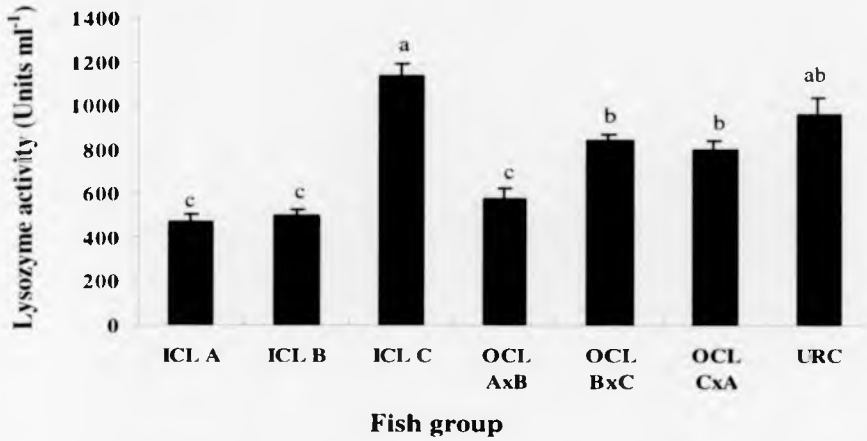


Figure 4.7 Serum lysozyme activity in the different groups of tilapia, *O. niloticus*

ICL A-Inbred clone 009356316, ICL B-Inbred clone 010036092, ICL C-Inbred clone 006812566, OCL AxB-Outbred clone AxB, OCL BxC-Outbred clone BxC, OCL CxA-Outbred clone CxA, URC-Unrelated control

Column with different letters (a, b, c) indicate significant difference at $P< 0.05$
Ten fish were sampled per group

phagocytose bacteria was highest in the ICL B and lowest in ICL A. It was found that the number of macrophages containing 0 bacteria in ICL B was significantly higher than in the ICL A, ICL C and OCL AxB fish. The URC group showed the second highest level, which was not significantly different from any other groups, of macrophages containing 0 bacteria. On the other hand, the number of macrophages containing ≥10 bacteria was highest in ICL C and lowest in ICL B with a significant difference of $P<0.05$ between them. All of the OCL groups had levels of phagocytosis intermediate between that of their parents, and these levels were not significantly different to each other (Figure 4.8). The estimation of initial number of total macrophages per g of head

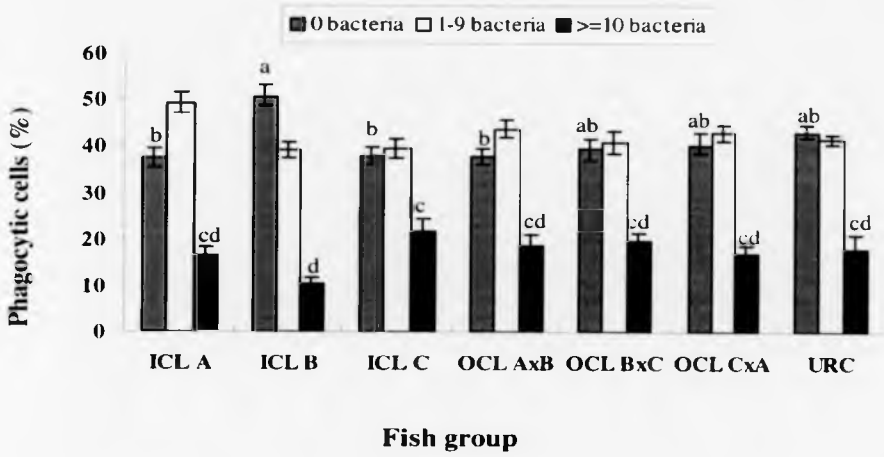


Figure 4.8 Level of phagocytic activity between macrophages of different groups of tilapia, *O. niloticus*

ICL A-Inbred clone 009356316, ICL B-Inbred clone 010036092, ICL C-Inbred clone 006812566, OCL AxB-Outbred clone AxB, OCL BxC-Outbred clone BxC, OCL CxA-Outbred clone CxA, URC-Unrelated control

Similar columns with different letters (a, b, c, d) indicate significant difference at $P<0.05$

Ten fish were sampled per group and 200 macrophages were counted per fish

kidney tissue in different groups showed that the ICL C had significantly higher ($P<0.05$) number of macrophages than all other groups except URC. Although, except ICL C, the total number of macrophages per g of kidney tissue was similar in all other groups, the calculated proportion of macrophages containing ≥10 phagocytosed bacteria showed considerable variation between groups. The ICL B and ICL C showed lowest and highest proportion of macrophages with ≥10 bacteria respectively and the OCLs had an intermediate proportion between that of their parents except OCL AxB which

showed slightly higher proportion than that of both parents (Table 4.3). The differential WBC and RBC counts did not show any significant difference between different groups of fish (Table 4.4).

Table 4.3 Serum lysozyme activity, number of macrophages per g of kidney tissue and phagocytosis of macrophages in different groups of tilapia, *O. niloticus*.

Fish group	Serum lysozyme activity (Unit ml^{-1})*	No. of macrophages g^{-1} of kidney tissue (x 10^8 cells)*	No. of macrophages contain phagocytosed bacteria (%) [r]			Calculated proportion of macrophages Containing ≥10 phagocytosed bacteria per g of kidney tissue*
			0 bacteria	1-9 bacteria	≥ 10 bacteria*	
ICL A	469.23 ± 36.20^{c}	4.71 ± 0.91^{b}	37.50± 1.78^{b}	46.13 ± 2.14	16.39 ± 1.75^{cd}	7.7 x 10^7 cells
ICL B	496.92 ± 31.03^{c}	5.98 ± 0.66^{b}	50.56 ± 2.63^{a}	39.06 ± 1.74	10.38 ± 1.41^{d}	6.2 x 10^7 cells
ICL C	1132.31 ± 60.60^{a}	10.46 ± 1.60^{a}	37.81 ± 2.13^{b}	39.38 ± 1.95	21.75 ± 2.49^{c}	2.3 x 10^8 cells
OCL AxB	575.39 ± 46.22^{c}	4.79 ± 0.85^{b}	37.88 ± 1.62^{b}	43.69 ± 1.94	18.44 ± 2.43^{cd}	8.8 x 10^7 cells
OCL BxC	836.93 ± 34.34^{b}	6.11 ± 1.14^{b}	39.44 ± 1.95^{ab}	40.94 ± 2.38	19.63 ± 1.77^{cd}	1.2 x 10^8 cells
OCL CxA	796.93 ± 43.56^{b}	5.86 ± 0.71^{b}	40.25 ± 2.58^{ab}	42.94 ± 1.76	16.81 ± 1.86^{cd}	9.9 x 10^7 cells
URC	958.46 ± 76.82^{ab}	6.71 ± 1.19^{a,b}	43.0 ± 1.55^{ab}	41.56 ± 1.12	17.81 ± 3.04^{cd}	1.2 x 10^8 cells

ICL A=Inbred clone 009356316, ICL B=Inbred clone 010036092, ICL C=Inbred clone 006812566, OCL AxB=Outbred clone 009356316 x010036092, OCL BxC=Outbred clone 010036092x006812566, OCL CxA=Outbred clone 006812566x009356316, URC=Unrelated control

Numbers with different superscript letters within a single column (a, b, c, d) indicate significant difference at $P<0.05$

Ten fish were sampled per group for all the immunological parameters

[r] 200 macrophages were counted per fish

* indicates significant correlation between serum lysozyme activity and phagocytosis of macrophages containing ≥10 bacteria, no of macrophages per g of kidney tissue, calculated proportion of macrophages containing ≥10 bacteria per g of kidney tissue at $P<0.005$, $P<0.01$ and $P<0.005$ respectively

Table 4.4 Haematocrit measurement, total and differential WBC counts, RBC counts in different groups of tilapia, *O. niloticus*.

Fish group	Haematocrit (%)	Differential WBC Counts ($x10^7$ cells ml^{-1}) [P]				Total no. of WBC ($x10^8$ ml^{-1})	No. of RBC ($x10^9$ cells ml^{-1})
		Neutrophil	Lymphocyte	Thrombocyte	Monocyte		
ICL A	30.1 ± 0.67[b]	0.25 ± 0.05	10.59 ± 0.45	2.53 ± 0.28	0.01 ±0.01	1.34 ± 0.05	2.07 ± 0.09
ICL B	33.9 ± 0.85[a]	0.25 ± 0.05	9.94 ± 0.58	2.79 ± 0.39	0	1.30 ± 0.06	2.31 ± 0.12
ICL C	28.4 ± 0.43[b]	0.22 ± 0.06	10.80 ±0.68	3.18 ± 0.35	0.01 ±0.004	1.42 ± 0.08	1.99 ± 0.11
OCL AxB	33.2 ± 0.51[a]	0.22 ± 0.06	9.10 ± 0.54	2.54 ± 0.18	0.02 ± 0.01	1.19 ± 0.05	2.04 ± 0.10
OCL BxC	33.4 ± 0.58[a]	0.42 ± 0.06	9.98 ±0.47	2.91 ± 0.37	0.01 ± 0.01	1.33 ± 0.07	2.25 ± 0.09
OCL CxA	36.3 ± 1.0[a]	0.51 ± 0.13	9.61 ± 0.65	4.10 ± 0.38	0.02 ± 0.01	1.42 ± 0.09	2.44 ± 0.14
URC	33.3 ± 0.96[a]	0.29 ± 0.09	9.24 ±0.67	3.09 ± 0.30	0.02 ± 0.01	1.26 ± 0.05	2.02 ± 0.10

ICL A=Inbred clone 009356316, ICL B=Inbred clone 010036092, ICL C=Inbred clone 006812566, OCL AxB=Outbred clone 009356316 x010036092, OCL BxC=Outbred clone 010036092x006812566, OCL CxA=Outbred clone 006812566x009356316, URC=Unrelated control

Numbers with different superscript letters (a, b) indicate significant difference at $P<0.05$

Ten fish were sampled per group for all the immunological parameters

[P] 200 white blood cells were counted per fish

4.3.2.3.2 Bacterial challenge

Fish injected with *A. hydrophila* started to die within 12 hrs after the injection. Fish mortality due to *A. hydrophila* first occurred in the ICL B which reached 50% after the first 2 days post injection (almost identical to the first trial) and cumulative mortality of 56.67% was found over the 6 days experimental period. Mortalities were also observed in other groups of fish, but at a lower level. Fish in the ICL A and ICL C showed only 3.33% (16.7% in first trial) and 6.67% mortality respectively over the experimental period, thus indicating a strong resistance to the challenge pathogen. The OCL AxB, OCL BxC and OCL CxA showed a similar trend in the level of mortalities,

which were 20%, 13.33% and 10% respectively. The cumulative mortalities of different groups of fish during the challenge period are presented in Figure 4.9. All dead fish showed typical symptoms of *A. hydrophila* infection described in Section 4.2.7.2.

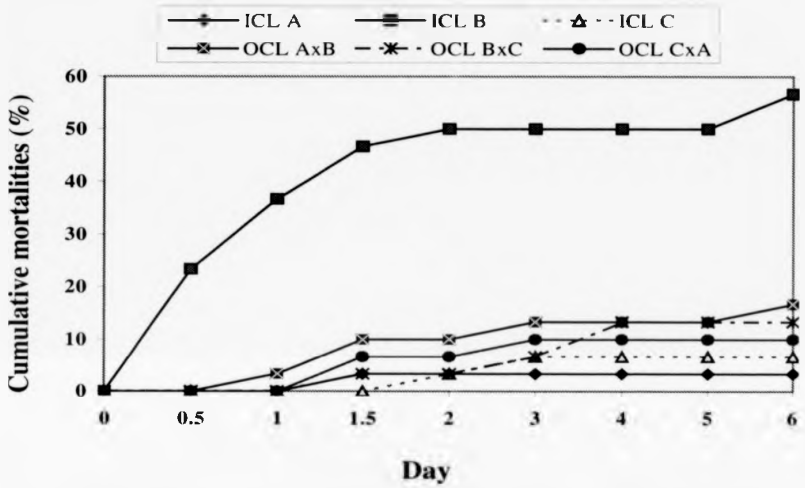


Figure 4.9 Cumulative mortalities (%) of tilapia during an artificial challenge by *A. hydrophila*

ICL A-Inbred clone 009356316, ICL B-Inbred clone 010036092
ICL C-Inbred clone 006812566, OCL AxB-Outbred clone AxB
OCL BxC-Outbred clone BxC, OCL CxA-Outbred clone CxA

Thirty fish were used for each group

At the end of the experiment (6 days after injection), all surviving fish were sampled for *A. hydrophila*. The re-isolation of bacteria showed that in the ICL groups, 16.67% of fish in the ICL B were infected with *A. hydrophila*, whereas in the OCL groups, 3.33% and 6.67% of the surviving fish in OCL BxC and OCL CxA respectively

carried the bacterium (Figure 4.10). Table 4.5 shows the susceptibility of different clonal groups of tilapia to *A. hydrophila*. Among all fish groups, the ICL B fish showed

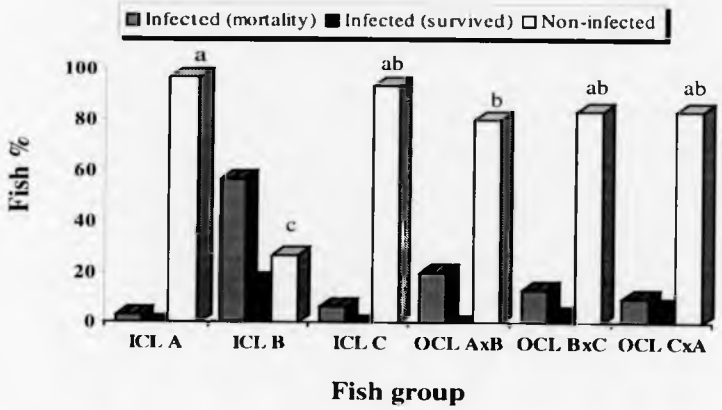


Figure 4.10 Susceptibility of tilapia to *A. hydrophila* after an artificial challenge

ICl A-Inbred clone 009356316, ICL B-Inbred clone 010036092, ICL C-Inbred clone 006812566, OCL AxB-Outbred clone AxB, OCL BxC- Outbred clone BxC, OCL CxA- Outbred clone CxA

Columns with different letters (a, b, c) indicate significant difference at $P<0.05$
Thirty fish were used for each group

Significant infection levels in different clonal lines are also presented in Table 4.5

more susceptibility to *A. hydrophila*, and the total number of infected (mortality plus survived) fish in ICL B was significantly higher ($P<0.05$) than all other fish groups. The OCL groups all showed *A. hydrophila* infection, but the number of fish infected was not significantly different between the groups. The level of infection in fish in the OCL AxB was significantly higher ($P<0.05$) than that of the ICL A and significantly lower

($P<0.05$) than the ICL B. In the OCL BxC, the total number of infected fish was significantly lower ($P<0.05$) from the ICL B but not from the ICL C. The OCL CxA did not show any significant difference for its infection level from any of the parental groups. No deaths or infection were found in the control groups injected with sterile PBS.

Each group of fish was maintained in three similar sized tanks (10 fish per tank) during bacterial challenge. Apparently a little intra-group variation was found in all the fish groups, but due to the small number of fish intra-group variations could not be analysed.

Table 4.5 Response of different clonal groups of tilapia, *O. niloticus* to an artificial bacterial challenge with *A. hydrophila*

Fish group	No of fish injected	Infected (mortality)	Infected (survived)	Non-infected
ICL A[a]	30	1	0	29
ICL B[c]	30	17	5	8
ICL C[ab]	30	2	0	28
OCL AxB[b]	30	6	0	24
OCL BxC[ab]	30	4	1	25
OCL CxA[ab]	30	3	2	25

ICl A-Inbred clone 009356316, ICL B-Inbred clone 010036092, ICL C-Inbred clone 006812566, OCL AxB-Outbred clone AxB, OCL BxC- Outbred clone BxC, OCL CxA - Outbred clone CxA

Fish groups with different superscript letters (a, b, c) indicate significant difference at $P<0.05$ for their levels of infection

4.4 Discussion

The non-specific immune response was compared between presumably genetically different groups of tilapia, *O. niloticus*, using an array of non-specific immunological parameters. Until now, little literature has been available on the immune response of tilapia (*O. niloticus*). Therefore, a preliminary study (Experiment 1) was carried out here to standardise assays for the non-specific immune responses of these fish as well as to determine which assays should be used in a larger study.

In the preliminary study, a clonal line, its related and unrelated control groups were used. During this study only one clonal line with sufficient number of individuals was available. The results of haematocrit, differential white blood cell counts, and respiratory burst of macrophages showed no significant difference between groups of fish. The serum lysozyme activity and phagocytosis of macrophages showed significant differences between the three fish groups, and there was a correlation between lysozyme activity and phagocytosis. The correlation co-efficient between increase in serum lysozyme activity and phagocytosis of macrophages with $\geq$10 phagocytosed bacteria was r= 0.49 ($P<0.05$, n=15). Macrophages with 0 and 1-9 phagocytosed bacteria did not show significant correlation with lysozyme. The lysozyme activity of the clonal line, related and unrelated control groups were significantly different ($P<0.05$) from each other. On the other hand, the phagocytic activity of these three groups did not show any significant difference between them for the first two categories of phagocytosis (i.e. 0, 1-9 phagocytosed bacteria) and only significantly higher ($P<0.05$) number of macrophages containing $\geq$10 bacteria was observed in the unrelated control group compared to other two groups. This type of categorisation of phagocytosis was previously used by Hardie *et al.* (1990) who observed that Atlantic salmon, *Salmo salar* maintained on diets with different amount of vitamin E produced different levels of

lysozyme activity and phagocytosis of macrophages. Fish groups fed with lower amounts of vitamin E showed higher levels of lysozyme activity and significantly higher number of macrophages with 1-9 phagocytosed bacteria compared to higher vitamin E fed group. In the ≥10 phagocytosed bacteria category, the opposite result was found. Leung *et al.* (1995) observed that macrophages activated with Freund's complete adjuvant (FCA) take up more *A. hydrophila* and faster than non-activated macrophages.

Members of the other two clonal lines ICL A, ICL B, and an URC were used for the bacterial challenge with a pathogenic strain *A. hydrophila* (T4). Amongst these three groups, ICL B appeared most susceptible to the bacterium although 16.7% and 41.7% fish from ICL A and URC groups died respectively in the first two days of the challenge due to *A. hydrophila* infection. The rest of the fish from these two groups survived without succumbing to infection. In contrast, 50% of fish from ICL B died over the first two days of injection and 33.3% were infected although they did not die. These fish were, however, infected with high levels of *A. hydrophila* at localised sites of the body, such as the eye, the peritoneum or the site of injection. Millar (1994) reported an intra-muscular injection of *A hydrophila* (T4) with a concentration of 3.2×10^6 cell ml^{-1} to tilapia, *O. niloticus* killed the fish within 18 hrs and produced severe focal lesions. A similar response by *O. niloticus* to *A. hydrophila* (T4) was observed with localised skin lesions progressing to fatality in a high proportion of the infected fish (Suthi, 1991). Angka *et al.* (1995) reported that the intra-muscular injection of *A. hydrophila* to catfish, *Clarias gariepinus* caused severe skin and muscle lesion at the injection sites of fingerlings, and fish began to die at 18 hrs after injection. Sharifpour (1997) observed that the intra-muscular injection of *A. hydrophila* (T4) at a concentration of 5.3×10^6 cells ml^{-1} caused infection in common carp, *C. carpio* and fish started to die within 12 hrs with one quarter of the injected fish dying between 12-24 hrs after injection.

Statistical analyses of the challenge results showed a significantly higher level of resistance of ICL A to *A. hydrophila* than ICL B. Since the two inbred clonal lines were genetically different, that is, they were homozygous for different allelic form of genes or different haplotypes of a gene, it might be argued that the particular allelic form or haplotype of genes in the ICL A were more effective at eliciting an immune response against the bacteria than the ICL B. The bacterial challenge used here reflects the non-specific immune response of the fish *in vivo*, while the MHC complex controls specific T-cell responses. The immunological studies performed *in vitro* to examine phagocytosis and lysozyme activity in clonal group of fish could not be compared directly with the challenge results since different clonal lines were used for the two studies, simply because insufficient fish were available to perform such analysis.

In the second experiment, three different inbred clonal lines (ICL A, ICL B, ICL C), three outbred clonal lines (OCL AxB, OCL BxC, OCL CxA) and an unrelated control group of tilapia were used. Based on the results obtained from the first preliminary experiment, the same non-specific immunological parameters were studied in the second trial with the exception of the respiratory burst assay. The respiratory burst by head kidney macrophages was not studied because the results obtained from the first experiment did not show any significant difference between the different groups of fish, and a poor level of consistency in the replicates within a fish group was found.

Both lysozyme activity and haematocrit values in the second study showed some significant differences between groups of fish, but there was no correlation between the two parameters. The ICL C showed significantly higher ($P<0.05$) levels of lysozyme activity than the ICL A and ICL B. The OCLs showed an intermediate response to that of their parents. The OCL BxC showed significantly higher ($P<0.05$) level of lysozyme activity than that of the ICL B and significantly lower ($P<0.05$) than the ICL C.

Similarly, the lysozyme activity in the OCL CxA was significantly higher ($P<0.05$) than that of the ICL A and significantly lower ($P<0.05$) than the ICL C. The intermediate lysozyme activity levels of the OCL BxC and OCL CxA to that of their parents suggest genetic effects for this trait. The lysozyme values of both OCL BxC and OCL CxA were closer to the mid-parent values which suggested additive parental effects in both the outbred clones. The OCL AxB showed a higher level of lysozyme activity (not significant) than those of both parents and this higher lysozyme activity suggesting likely positive heterosis in the outbred clones. Grinde *et al.* (1988) reported a wide variation in kidney lysozyme activity between different fish species and within a species. They observed that rainbow trout in different localities had 5 to 10 fold intra-species variation in lysozyme activity which suggested an existence of a genetic influence on the lysozyme levels. The haematocrit values of the OCL AxB and OCL BxC lay somewhere between that of their parents. The heamatocrit value of the OCL AxB was significantly higher ($P<0.05$) than that of ICL A and similar to ICL B. Likewise, the OCL BxC showed significantly higher haematocrit value than the ICL C and similar to ICL B. The OCL CxA did not show any similarity with that of their parental haematocrit values and it was significantly higher ($P<0.05$) than both ICL A and ICL C. The haematocrit values of the OCL AxB and OCL BxC were nearer to that of the common parent ICL B than the ICL A and ICL C respectively which suggested dominant effect of the ICL B on the outbred crosses.

In phagocytosis, the phagocytic activity of macrophages were classified into three categories, macrophages with 0 bacteria, 1-9 bacteria and $\geq$10 bacteria. The phagocytic activity of all fish groups showed a correlation with their serum lysozyme activity. The correlation co-efficient between serum lysozyme activity and phagocytosis of macrophages containing $\geq$10 phagocytosed bacteria was $r=0.38$ ($P<0.005$, $n=56$).

Macrophages containing 0 and 1-9 phagocytosed bacteria did not show significant correlation with the lysozyme activity. The ICL C group exhibited the highest level of lysozyme activity and at the same time had the highest number of macrophages with ≥10 phagocytosed bacteria. Since macrophages produce lysozyme this result indicated that the higher phagocytic activity of macrophages resulted in an increased production of lysozyme. The macrophage phagocytic activity of the OCLs fish showed correlation with their lysozyme activity and demonstrated an intermediate phagocytic activity in all three categories to that of their parental groups. The phagocytic activity (≥10 phagocytosed bacteria) of macrophages of the OCLs also revealed individual parental genetic influence on the offspring. The OCL AxB showed higher phagocytic activity (not significant) than the both parents which suggesting likely positive heterosis in the outbred clones. The phagocytic activity of OCL BxC was nearer to ICL C parent than ICL B which suggested that ICL C parent likely elicited dominant effect on the phagocytic activity of its outbred offspring. The phagocytic activity of the macrophages in the OCL CxA was closer to ICL A parent's value than ICL C which suggested that ICL A had likely dominant genetic effect in its outbred clone, OCL CxA.

The total number of macrophages per g of head kidney tissue in all fish groups showed a significant correlation with their serum lysozyme activity ($r=0.84$, $P<0.005$, $n=7$). When the phagocytic activity of macrophages in terms of number containing ≥10 bacteria was proportionately calculated with total number of macrophages per g of kidney tissue, it also showed a highly significant correlation with lysozyme activity ($r=0.89$, $P\leq0.005$, $n=7$). The proportionate number of macrophages in the ICL groups showed large interclone variation: the value for the ICL C was about 3 and 4 times higher than those of the ICL A and ICL B respectively. The variation in the proportionate number of macrophages which phagocytosed ≥10 bacteria in different ICL

groups might be due to the degree of effectiveness of their non-specific defence mechanisms. But when the outbred clones were produced by crossing between inbred lines, two out of the three outbred lines showed a clear parental genetic influence on the offspring. The OCL BxC and OCL CxA showed an intermediate value to that of their parents whereas the OCL AxB showed higher value than those of their parents.

The aforementioned inbred and outbred clonal lines were also used for the bacterial challenge. The inbred clonal lines, ICL A and ICL B had previously been used in the first challenge experiment, so there was an opportunity to confirm the response obtained with the second challenge. Fishes injected i.p. with *A. hydrophila* (T4 strain) suspension ($5x10^6$ cells ml^{-1}) started to die within 12 hrs. The ICL B showed the highest mortality confirmed as being due to *A. hydrophila* infection, whereas some mortalities were also occurred in the ICL A and ICL C. As well as the fish which died in the ICL B during the challenge, some of the survivors showed *A. hydrophila* infection. No bacteria were re-isolated from fish in the ICL A and ICL C. Similar results of mortality and infection with ICL B fish have also been found in the first challenge experiment, while the ICL A fish showed fewer mortalities than in the first challenge. Liu *et al.* (1990) obtained experimental infection of tilapia with *A. hydrophila* ($7.5x10^5$ cells ml^{-1}) by means of immersion or oral inoculation. Four days after inoculation, the fish started to die and showed marked hyperemia and petechial haemorrhage on the body surface, base of fins and the operculum.

The outbred clonal lines, OCL AxB and OCL BxC showed significantly higher resistance to *A. hydrophila* compared to their disease susceptible parental inbred clonal line ICL B. The OCL AxB, OCL BxC and OCL CxA showed 20%, 13.33% and 10% mortality respectively, and the surviving fish in OCL BxC and OCL CxA showed 3.33% and 6.67% infection with *A. hydrophila* respectively. The number of infected fish in the

OCLs did not show any significant difference. Wiegertjes *et al.* (1993) characterized two brood females, R8 (Hungarian origin) and W (The Netherlands origin) of *C. carpio* in terms of resistance or susceptibility to disease by a bath challenge with *A. salmonicida*. They used the offspring of these two females including gynogenetic offspring w produced from the W female and r8 progeny from a normal cross between a male and female of R8 line and obtained 100% survivality in the w and 25% mortality in r8 progenies. These results indicated that the W female brood was homozygous resistant (RR) and thus the gynogenetic progenies from this female were 100% resistant. In contrast, the parents of r8 progenies were assumed to be heterozygous (Rr) and as a result the r8 progenies were 25% susceptible to *A. salmonicida*. This progeny testing result identified the two female broods' (R8 and W) disease resistance who might produce disease susceptible and resistant progeny respectively upon gynogenetic reproduction. In the present study, ICL B was the most susceptible to *A. hydrophila* infection, but the cross breeding of ICL B with other inbred lines, ICL A and ICL C produced more disease resistant hybrids, AxB and BxC than the ICL B. Kincaid (1983) reported that quantitative traits associated with reproduction or physiological efficiency are often affected by inbreeding depression, such as growth rate and survival, which in turn might improve in hybrid lines. Nagy *et al.* (1984) observed fast growth in hybrids produced from two gynogenetic carp lines. Other workers have indicated that hybrids have superior viability and disease resistance (Bakos, 1987, Ilyassov, 1987).

The inbred clonal lines used in this study were genetically different i.e. they were different from each other for a particular allelic form of genes. For example, in Chapter 3 of this thesis, MHC class II B genotypes of these lines were determined which showed that they have different MHC class II B haplotypes. During bacterial challenge, the ICL B showed a significantly higher number of infected fish compared to ICL A and ICL C.

It is suggested from the challenge results that the genetic variations might cause different non-specific immune response in different lines and as a result, variations in disease resistance can be found between populations. The bacterial challenge of the clonal lines showed that there was a relationship between the investigated immune parameters and susceptibility to *A. hydrophila* infection (Table 4.6). The ICL A and ICL B did not show significant differences for their lysozyme activity, number of macrophages per g of kidney tissue and phagocytosis of macrophages containing 1-9 and ≥10 bacteria, but ICL B showed significantly higher susceptibility to *A. hydrophila* than ICL A. The probable reason for the higher susceptibility of the ICL B might be due to it possessing a significantly higher number of macrophages which were unable to phagocytose bacteria than that of ICL A. The ICL B also showed lower number of proportionately calculated macrophages than that of the ICL A. On the other hand, the ICL C showed significantly higher lysozyme activity, number of macrophages per g of kidney tissue and phagocytosis of macrophages containing 1-9 and ≥10 bacteria than those of the ICL B. The ICL C also possessed about 4 times the number of macrophages which phagocytosed ≥10 bacteria than the ICL B. Since the ICL C showed a significantly lower level of susceptibility to *A. hydrophila* than ICL B, it might be assumed that during bacterial challenge the higher number of macrophages in the ICL C led to comparatively higher phagocytic activity against the challenge bacteria. However, the ICL C and ICL A did not show significant difference for their disease resistance although ICL C possessed about 3 times more macrophages which phagocytosed ≥10 bacteria than ICL A.

The infection levels of the OCL AxB and OCL BxC were intermediate to that of their parents. The OCL AxB showed significantly higher infection than ICL A and

Table 4.6 Summary of results of non-specific immune response and susceptibility to *A. hydrophila* infection of different clonal groups of tilapia, *O. niloticus*

Fish group	Serum lysozyme activity (Unit ml^{-1})	No. of macrophages g^{-1} of kidney tissue (x 10^8 cells)	No. of macrophages contain phagocytosed bacteria (%)			Calculated proportion of macrophages containing ≥10 phagocytosed bacteria per g of kidney tissue	Haematocrit (%)	Susceptibility of tilapia to *A. hydrophila* infection (%)		
			0 bacteria	1-9 bacteria	≥ 10 bacteria			Infected (mortality)	Infected (survived)	Non-infected
ICL A	469.23± 36.20^{c}	4.71 ± 0.91^{b}	37.5 ± 1.78^{b}	46.13 ± 2.14	16.39 ± 1.75^{cd}	7.7 x 10^7 cells	30.1 ± 0.67^{b}	3.33	0	96.67
ICL B	496.92 ± 31.03^{c}	5.98 ± 0.66^{b}	50.56 ± 2.63^{a}	39.06 ± 1.74	10.38 ± 1.41^{d}	6.2 x 10^7 cells	33.9 ± 0.85^{a}	56.67	16.67	26.67
ICL C	1132.31 ± 60.60^{a}	10.46 ± 1.60^{a}	37.81 ± 2.13^{b}	39.38 ± 1.95	21.75 ± 2.49^{c}	2.3 x 10^8 cells	28.4 ± 0.43^{b}	6.67	0	93.33
OCL AxB	575.39 ± 46.22^{c}	4.79 ± 0.85^{b}	37.88 ± 1.62^{b}	43.69 ± 1.94	18.44 ± 2.43^{cd}	8.8 x 10^7 cells	33.2 ± 0.51^{a}	20	0	80
OCL BxC	836.93 ± 34.34^{b}	6.11 ± 1.14^{b}	39.44 ± 1.95^{ab}	40.94 ± 2.38	19.63 ± 1.77^{cd}	1.2 x 10^8 cells	33.4 ± 0.58^{a}	13.33	3.33	83.33
OCL CxA	796.93 ± 43.56^{b}	5.86 ± 0.71^{b}	40.25 ± 2.58^{ab}	42.94 ± 1.76	16.81 ± 1.86^{cd}	9.9 x 10^7 cells	36.3 ± 1.0^{a}	10	6.67	83.33

ICL A=Inbred clone 009 356 316, ICL B=Inbred clone 010 036 092, ICL C=Inbred clone 006 812 566, OCL AxB=Outbred clone 009 356 316 x010 036 092, OCL BxC=Outbred clone 010 036 092x006 812 566, OCL CxA=Outbred clone 006 812 566x009 356 316

Numbers with different superscript letters within a single column (a, b, c, d) indicate significant difference at $P<0.05$

significantly lower than ICL B which indicated dominant effect of ICL A in the outbred offspring. The OCL BxC, on the other hand, showed significantly lower infection than ICL B and higher infection than ICL C. This result suggested that the expression of the ICL C inherited genes in OCL BxC was dominant. The OCL AxC showed higher infection than the both parents but not significantly different from any of them. Although all the OCL groups were infected by *A. hydrophila*, they did not show significant differences from each other. They demonstrated similar response to the challenge bacteria and therefore, none of the groups was superior to others. The analysis of the genetic inheritance in the OCLs showed that during crossing between a high and low resistant parent, the high resistant parent had a dominant effect in the inherited genes and thus the resulting progenies became more resistant than the low resistant parent. These results indicated that there might be a possible way to produce more disease resistant individuals through segregation of resistant alleles from a disease resistant population by crossbreeding. Hines *et al.* (1974) reported that all F1 crosses between four inbred strains of common carp were unaffected by the infections, although two of the inbred strains were previously susceptible to disease.

Response of a naïve fish to a pathogen or parasite is entirely non-specific. When an artificial bacterial challenge is carried out with an unimmunized fish, its non-specific defence components such as lysozyme, complements, macrophages, granulocytes encounter the bacteria and kill it. In the killing of bacteria, the phagocytic cells mainly macrophages play an important role to breakdown the bacteria into peptides and eliminate them. The non-specific immune response varies between individual fish and therefore, during artificial bacterial challenge different fish responds differently. In case of an immunised or pre-treated fish, the immune response to the challenged pathogen is specific. The specific cellular defence components such as T lymphocytes, B

lymphocytes can easily recognise the pathogen along with MHC, respond quickly by proliferating their clonal populations and kill the pathogen. From the above discussion, it can be concluded that there is a relationship between disease resistance and genetic variation for the studied lines of *O. niloticus*.

Chapter V

General discussion

5. General discussion

All of the experiments conducted in this thesis were designed with a common objective, to examine the genetic variation in the specific and non-specific immune responses of different clonal groups of tilapia, *O. niloticus*. For this study, MHC class II B genes are considered as a variable unit of genetic composition between tilapia groups. Therefore, homozygous inbred and clonal lines were produced on the basis of different MHC class II B genotypes although it is recognised they may be the same or different at other parts of the MHC or other loci.

Inbred strains are valuable tools for various genetic studies. Inbred lines are homozygous at every gene locus because they result from duplication of a haploid set of homozygous maternal or paternal chromosomes. Clonal lines produced from different families may be fixed for different alleles of polymorphic genes, so they are different from each other. Each clonal line may have a distinct response to a stimulus, and for this reason, any kind of biological assays can be conducted with them. Conventional inbred strains have only been developed for two species of fish, *Xiphophorus maculatus* (Kallman, 1970) and *O. latipes* (Hyodo-Taguchi, 1980). Some fish such as the particular cold water fish salmonids and cyprinids have long generation times and therefore, conventional full-sib mating is not an ideal method for producing inbred strains (Falconer, 1981). Rapid chromosome manipulation techniques such as gynogenesis have been applied to these species to produce inbred strains (Thorgaard, 1983; Nagy and Csanyi, 1984). Since tilapia has a comparatively short generation time, gynogenesis is a suitable method for producing inbred meiotic and mitotic individuals from this species. Early heat shock is applied for meiotic gynogenesis to induce retention of the second polar body, while late heat shock is applied to suppress the first mitotic division during mitotic gynogenesis. Gynogenetic inbred strains and their

crosses can be used for standardisation of bio-assays (Falconer, 1981; Richter *et al.*, 1987), for studies of immune response (Kaastrup *et al.*, 1989), sex determination and differentiation (Komen and Richter, 1993) and for stock improvement (Wilkins, 1981; Gjerde, 1988).

Although both meiotic and mitotic gynogenetics were successfully produced in this study, their survival rates were low especially in the mitotic gynogen population. The mean survival rates of meiogynes and mitogynes were 48.95 ± 7.35% and 6.88 ± 1.53% respectively at the yolk sac resorption stage, compared to control. Yields of meiogynes ranging from 0 to 63% have been reported in *O. niloticus* (Chourrout and Itskovich, 1983; Mair *et al.*, 1987; Mair, 1988; Penman, 1989; Hussain *et al.*, 1993). An average 24% survival in the meiogynes was obtained at the first feeding stage by using pressure shock of 8,000 p.s.i. for 2 mins commencing 9 mins after fertilisation (Mair, 1988). The low survival of meiogynes might be due to the expression of recessive deleterious and lethal genes resulting from their homozygosity. Unlike meiogynes which are partially heterozygous due to recombination between non-sister chromatids, mitogynes are completely homozygous for every gene locus. Therefore, the presence of homozygous recessive deleterious and lethal alleles should be higher in mitogynes than meiogynes. As a result, the survival rate of mitogynes is lower than that of meiogynes. Low survival rates of tilapia mitogynes have been reported with 2.0% survival in *O. niloticus* (Hussain *et al.*, 1993) and 0.8 and 0.8% survival in *O. aureus* and *O. mossambicus* respectively (Mair, 1988). As well as the lower survival rates observed in the gynogens, higher frequencies of homozygotes for deleterious alleles cause deformities in diploids. Don and Avtalion (1988a), and Varadaraj (1990) reported that an increased loading of deleterious genes was responsible for the higher proportion of deformities seen among the gynogens compared to control fish.

A number of factors involved in the chromosome-set manipulation may also affect the survival rate of gynogens. In most of the studies with tilapia, naturally ovulated eggs were collected from females maintained in laboratory aquarium, where they released eggs under normal courtship with males. Some mechanical damage may occur to the eggs during their collection from the aquarium. Artificial stripping is also used to collect eggs and this technique is completely dependent on manual selection of ovulated females. Stripping of eggs prior to complete ovulation results in unacceptably low levels of fertilisation (Mair, 1988). After *in vitro* fertilisation, the eggs need to be incubated in a water system which provides optimum water quality (Rana, 1988; Yeheskel and Avtalion, 1988). The system requires clean, pathogen-free water preferably supplied with antibiotic treated or UV-sterilised water (Subasinghe and Sommerville, 1985; Don *et al.*, 1987). Inactivation of sperm by higher dosages of UV irradiation, or deleterious effects of non-specific physical shocks applied to the eggs may also result in low survival of gynogens. For example, the heat shock is close to the upper lethal limit of tilapia eggs which may affect the zygote and blastula development (Subasinghe and Sommerville, 1992).

Analysis of the sex ratio of fish in this study showed a high proportion of males in both the meiotic and mitotic gynogenetic groups of a single family. Fish produced from three other families were mostly female in both gynogenetic groups. Higher proportions of males in mitogynes might arise from the effects of rare autosomal sex determining genes. The occurrence of a higher percentage of gynogenetic males in *O. niloticus* was also reported by Hussain *et al.* (1994a). They suggested that a sex determining locus (SDL-2) with two alleles might cause sex reversal from female to male under the recessive homozygous condition. Mair *et al.* (1991a) observed a considerable proportion of males in the inbred meiogyne group of *O. niloticus*. They

also found a higher proportion of males (20%) in the inbred mitogyne group. From these results they assumed that the mitotic males might be produced from a natural sex reversal mechanism that develops in the homozygous condition of rare autosomal, recessive, sex influencing genes.

Mitotic gynogenetic females were used to produce inbred and outbred clones. Inbred clones were produced from mitotic females by meiotic gynogenesis. The resultant progenies are expected to be all female. Neomales (XX) were produced by administering hormone (17 α-methyl testosterone) treatment. The resulting neomales were used to firstly produce inbred clones by crossing them with their mitotic mother, and secondly to produce outbred clones by crossing with another mitotic female. The highest number of inbred and outbred clones of *O. niloticus* produced to date has been made in the study. Thirteen inbred clonal lines, including 8 sibling clonal lines from 5 different families, and several outbred clonal lines were produced. There are several ways by which the inbred and outbred clonal lines can be produced. These are as follows :

i) Eggs from mitotic gynogenetic females can be fertilised with UV irradiated sperm and early heat shock (41-42 °C for 4 mins at 5 mins post fertilisation) can be applied to induce retention of the second polar body during the second meiotic division, and the resultant offspring are called inbred clones. These clones will carry only maternal chromosomes and they will have no paternal inheritance. Their absolute maternal inheritance can be confirmed by DNA fingerprinting.

ii) Inbred clones can be produced by fertilising eggs from an androgenetic female (XX) with UV irradiated sperm, followed by early heat shock for the retention of the second

polar body. DNA fingerprinting can be used to confirm complete UV irradiation of sperms and no paternal inheritance in the clonal offspring.

iii) UV irradiated eggs can be fertilised with sperm collected from androgenetic males (YY) followed by late heat shock (42-42.5 °C for 4 mins at 25-27 mins post fertilisation) to suppress the first mitotic division. Clones produced in this way are called inbred clones. DNA fingerprinting can also be used to ensure complete UV irradiation of eggs and the clones will have only paternal bands and no maternal bands.

iv) Conventional sib mating between gynogenetic cloned females and neomales, or crossing between neomales and their mitotic mother can produce inbred clones.

v) Conventional sib mating between androgenetic cloned males (YY) and neofemales (YY), or crossing between neofemales and their androgenetic father can produce inbred clones.

vi) Eggs from gynogenetic cloned females, or mitotic gynogenetic females from one family can be fertilised with sperm collected from neomales from another family. The resultant progenies are called outbred clones.

vii) Outbred clones can also be produced by fertilising eggs from neofemales (YY) in one family with sperms of androgenetic cloned males from another family.

Although homozygous inbred lines and their clones were successfully produced in this study, it was necessary to verify their homozygosity, that is, their parental

inheritance. The homozygosity of the inbred strains may be determined by comparing allozymes, but this does not provide as much information as multilocus DNA fingerprinting. Allozyme studies can, however, be carried out as a primary test to examine homozygosity of the inbred lines. It is comparatively easy, quick and less expensive than DNA fingerprinting.

In multilocus DNA fingerprinting, hypervariable DNA probes are used to detect multiple minisatellite loci throughout the genome, the results of which represent high levels of polymorphism of the fingerprint loci. The fingerprints were generated using Jeffreys 33.15 hypervariable probe (Jeffreys *et al.*, 1985). This probe is composed of 10-15 bp, G-C rich tandem repeat sequences. These fingerprints successfully showed parental inheritance in the gynogenetic offspring, with all meiotic and mitotic gynogenetics possessing only the maternal bands, and none of them had the paternal specific bands. The meiotic gynogens retain some heterozygosity, particularly at loci distal to the centromere, whereas the mitotic gynogens are completely homozygous for every gene locus. Thus it is expected that the DNA profiles of meiogynes should contain more bands than the mitotic gynogens. The fingerprints of inbred clones showed that all bands were identical with those of their mitotic mother, but no bands came from their father, while all outbred clones were identical and shared all bands from both parents. Therefore, multilocus DNA fingerprinting can be used in the analysis of gynogenesis as previously shown by Georges *et al.* (1988). In the case of androgenetic clones, the fingerprints showed all bands came from father and there were no maternal bands in any of the clonal individual.

DNA fingerprinting produces highly variable, genetically distinct markers for individuals (Fields *et al.*, 1989). These individual-specific markers provide a new source of genetic indicators that can be used in parentage (Westneat, 1990; Rico *et al.*, 1991)

and linkage analysis (Jeffreys *et al.*, 1986), forensics (Bär and Hummel, 1991), and genetic diversity studies (Reeve *et al.*, 1990). It can also be used to identify gynogenetic and androgenetic individuals and clones. High levels of polymorphism in the minisatellite loci detected by hypervariable DNA probes are not usually meaningful when comparing outbreeding wild populations of fish. However, in some species which have low levels of genetic variation or populations that have experienced bottleneck effects, genetic variation of these species can be determined through population-level comparisons using these multilocus DNA fingerprinting techniques. For the stocking of commercially important fish, highly variable loci in DNA fingerprints may produce additional markers that can be used to evaluate inbreeding depression, especially when used along side other techniques such as allozyme and mtDNA analysis.

The identification of MHC loci by PCR, like DNA fingerprinting, potentially provides information about the inheritance of fish resulting from gynogenesis. Amplification of MHC class II B genes in this study, using two specific primers, identified multiple loci in the chromosome. The number of amplified loci varied from one individual to another. Screening of MHC class II B inheritance in the meiotic and mitotic gynogens showed that both types of gynogens possessed only maternal bands, and the mitotic gynogens were homozygous for one or other maternal alleles. These mitotic gynogens were therefore considered as homozygous for certain MHC haplotypes. PCR using specific MHC primers can be considered as a potential technique for identifying different MHC haplotypes in the clonal lines. Since the clones were produced from a mitotic gynogenetic female in this study they had identical haplotypes to those of their mother.

The clonal lines used in this study were identified fixed for different MHC class II B haplotypes, but their homozygous nature for other MHC genes was unknown. So

far, 21 MHC class II B haplotypes have been identified in *O. niloticus* (Malaga *et al.* in preparation), but their functional expression is yet to be elucidated. Since the number of MHC loci varies between haplotypes, the functional expression of each haplotype might be specific. To examine the role of the MHC haplotypes, reciprocal scale grafting was conducted between three clonal groups shown to have different MHC class II B genotypes. All transplanted grafts were acutely rejected by the recipient fish groups. This therefore indicated that the homozygous MHC class II B haplotypes of the clonal groups were functionally different from each other and resulting in graft rejection. But it should be noted that apart from MHC class II B genes, some other MHC genes also might be involved in this graft rejection which was unknown. Similar results were reported by Komen *et al.* (1990) when skin grafts reciprocally exchanged between members of the two homozygous inbred strains of common carp were also acutely rejected.

Fish have both specific and non-specific immune defences. Rejection of grafts results from response of the specific defence mechanisms. In fish, transplantation immunity has been studied using mainly scale grafting (reviewed by Botham *et al.*, 1980). The rejection profiles of scale transplants can be used to determine the degree of inbreeding in gynogenetic tilapia. This technique is very simple, quick and permits simultaneously testing of a large number of grafts. However, the technique may not have sufficient resolution to differentiate between controls, meiogynes and mitogynes (Avtalion *et al.*, 1988). Allograft rejection occurs by the cumulative response of major and minor histocompatibilities. The major and minor histocompatibility loci are thought to be polymorphic. Therefore, to determine the relative contribution of the major and minor histocompatibility loci in graft rejection, it is necessary to reduce the heterozygosity of these MHC loci. In this respect, gynogenesis is a very effective

method, which can greatly reduce the allelic diversity at a given gene locus (Roux and Volpe, 1975; Cherfas, 1981; Hussain *et al.*, 1993). Clonal lines used for scale grafting in this study were gynogenetically produced and they were fixed for different MHC class II B haplotypes. During scale grafting all clonal lines respond positively and their rejection of allografts indicated an MHC control specific cellular immune response against foreign antigens. Kallman (1970) used naturally occurring gynogenetic fish, *Poecilia formosa* for allograft experiments, but his studies did not immediately initiate the general use of gynogenesis to elucidate the nature of the histocompatibility system in fish. In a similar experiment using *X. laevi*, Du Pasquier *et al.* (1977) provided evidence for the XLA-complex, the MHC homologue in this species.

The first and often the most important response of fish to infectious agents is non-specific immunity. It includes the soluble and cellular elements of acute inflammation, various serum proteins such as lysozyme, complement proteins, and the circulating and tissue phagocytes (Trust, 1986; Blazer, 1991). In order to determine the variation of non-specific immunity between groups of cloned tilapia in the current study, an array of immunological parameters such as serum lysozyme activity, respiratory burst and phagocytosis of head kidney macrophages were studied. Besides these, haematological tests such as total leucocytes and erythrocytes counts, differential leucocyte counts and haematocrit measurements were conducted.

Haematological tests and analysis of serum constituents are useful indicators in the diagnosis of disease in fish, but it must be remembered that some factors such as age, strain, nutritional state, season, environmental stress (e.g. crowding, density, water quality) and sexual maturity can influence the results obtained (see Sandnes *et al.*, 1988). Haematological measurements of fish are also influenced by temperature (Lane, 1979; Houston, 1980; Dunn *et al.*, 1989; Lie *et al.*, 1989b). Blaxhall and Daisley (1973)

suggested that routine haematological tests could help to assess the health and stress status of fish. They observed that the normal physiological range of fish is wider than that of humans.

It is known from the literature that the number of lymphocytes in blood can vary between individuals of a single species, depending on the conditions under which the blood sampled, for example, on the physiological conditions of the fish (Klontz, 1972). Watson *et al.*, (1963) reported that goldfish lymphocytes consists of about 30% of the total blood leucocytes. They also claimed 70% of all leucocytes are thrombocytes. The ratio of lymphocytes to thrombocytes in rainbow trout, *O. mykiss* blood has been reported as 25:1 by McCarthy *et al.*, (1973), while Weinreb (1958) claimed the ratio was 2:1. Little information is available on the haematology of tilapia. However, the present study showed a similar lymphocyte to thrombocyte ratio (3:1 ratio) to that observed in rainbow trout (Weinreb 1958). It also showed a variation in the number of lymphocytes and thrombocytes within and between fish groups but the differences were not significant. Gardener and Yevich (1969) observed seasonal variation in the number of lymphocytes and thrombocytes in cyprinodonts, where 2-13% lymphocytes were counted in winter and 7-8% in summer, and thrombocytes varied between 82-95%. Haematocrit measurements are normally used to check anaemia in fish. The haematological values obtained in this study ranged from 28.4% to 36.3% which is similar to haematocrit value observed in the same species (33%) (Mohammed Al-Owafeir, personal communication) and also similar to other haematocrit values reported in tropical freshwater fishes such as 29% in Indian carp (Raizada and Singh, 1982) and 30% in brown snakehead (Natarajan, 1981). Although all the fish groups were reared in an identical condition, the haematocrit values showed significant differences between

fish groups. These haematocrit differences might indicate some kind of genetic influence on it.

Serum- and phagocyte-mediated killing are the two major defence mechanisms of non-specific immunity in fish (Trust, 1986; Blazer, 1991). Fish serum exhibits haemolytic ((Ingram, 1980) and bactericidal (Kawakami *et al.*, 1984) activities by complement-like proteins, both through the classical and alternative pathways (see reviews by Ingram, 1980, 1990). Serum lysozyme splits the peptidoglycan layer of the bacterial cell wall, particularly Gram-positive bacteria and thus facilitating cell wall lysis (Chipman and Sharon, 1969). It is believed that macrophages and granulocytes are the main producers of lysozyme. In mammals, lysozyme exhibits many functions such as bacteriolysis, opsonization, antineoplastic activity, and immune response potentiation (Lie and Syed, 1986).

Lysozyme has been detected in the blood, kidney, spleen, mucus and some other parts of the fish body. Its level of activity varies between fish species (Lie *et al.*, 1989a). The levels of lysozyme concentration in fish increase during infection or injection with foreign particles (Fletcher and White, 1973; Siwicki and Studnicka, 1987). Ingram (1980) suggested that lysozyme is more important for fish than for mammals, since fish apparently possess a less developed specific immune system. In the present study, lysozyme activity was found to vary between the different clonal groups of tilapia with one inbred clonal line (ICL C) showing significantly higher levels of lysozyme activity than other two inbred lines (ICL A and ICL B). The outbred clones showed an intermediate level of lysozyme activity to that of their parents. Since the clonal lines were different from each other for a particular set of alleles, their differential lysozyme activity levels might be suggested a genetic influence on it. The lysozyme activity levels

of outbred clones indicated dominant or additive parental genetic effects on their offspring.

Macrophages provide a major line of defence against invading microbes. They are the first cells to respond to any kind of inflammation, and migrate in large numbers to the sites of inflammation. During an inflammatory response macrophages become activated which results in them increasing in size, undergoing metabolic changes and developing increased phagocytic and killing properties. Macrophages appear to be the dominant infiltrating cells in most cellular inflammatory responses in teleosts and are capable of developing into epitheloid cells and multinucleated giant cells (Roberts, 1989). Their stimulation and functional response is non-specific, but they affect and are affected by products and cells of the immune system (Ellis, 1980, 1981).

In tilapias, phagocytes which includes macrophages and neutrophils, have similar phagocytic activity (Suzuki, 1986). It is known from the present study that the intensity of phagocytic activity of macrophages varies from one group of fish to another and it has a correlation with the serum lysozyme activity. The clonal line ICL C showed higher lysozyme activity and also higher level of phagocytic activity of macrophages. There is no direct influence of lysozyme on phagocytosis of macrophages. But since macrophages produce lysozyme which helps to break down the cell wall of bacteria, a higher level of phagocytic activity of macrophages produces higher amounts of lysozyme. The present study showed that total number of macrophages per g of kidney tissue was significantly different from one group to another. During phagocytosis some of the clonal lines did not show significant difference for their phagocytic activity of macrophages. But when the phagocytic activity of macrophages was totally estimated with the total number of macrophages per g of kidney tissue, the calculated value showed a big difference between groups. Therefore during comparing phagocytic

activity of macrophages between populations, the total number of macrophages should be considered. The phagocytic activity of macrophages in different inbred clonal lines showed significant differences between lines and the outbred clonal lines showed an intermediate level of phagocytic activity to that of their parents. Therefore the different levels of phagocytosis in inbred clonal lines suggested a differential genetic influence on phagocytic activity of macrophages and dominant or additive genetic effects of the parents on the outbred offspring.

During phagocytosis macrophages move towards the microbial molecules guided by a gradient of chemotactic molecules. Once particles are surrounded by the surface of the macrophage, the plasma expands along the surface of particles and engulfs them in a phagosome. This process requires the active participation of actin-containing microfilaments present under the cell surface. After engulfment of bacterial particles by macrophages, it has been shown that the phagosomes fuse with primary lysosomes by forming secondary lysosomes (phagolysosomes) (Nichols *et al.*, 1971). Degradation of the ingested bacteria occurs within the lysosome, which contains hydrolases and other enzymes essential for digestion of the bacteria. Sometimes macrophages engulf bacteria but are unable to kill them. Many human pathogens such as *Mycobacterium*, *Yersinia*, *Salmonella* and *Legionella* are known to be capable of evading or resisting intracellular killing by phagocytes (Moulder, 1985; Finlay and Falkow, 1989).

During bacterial challenge with a pathogenic *Aeromonas hydrophila* strain, a significant variation of disease resistance was found between the different clonal groups of fish in this study (Chapter 4). Prior to challenging the clonal fish, an optimum bacterial dose was determined through a preliminary challenge to produce sufficient infection with minimum mortalities. A bacterial suspension with a concentration of 5×10^6 cells ml^{-1} was intraperitoneally injected into the fish. The dead fish which resulted

from the challenge showed typical clinical signs of *A. hydrophila* infection including marked hyperemia and petechial haemorrhage on the body surface and base of fins; reddened anus, swollen abdomen and sometimes swelling at the injected area.

Table 5.1 shows the immunological status of different clonal lines and the influence of non-specific immune responses on disease resistance. The challenge results

Table 5.1 Sex and immunological status of different clonal lines of tilapia, *O. niloticus*

Clonal lines*	Sex ratio (%)		Rate of graft rejection		Lysozyme activity	Phagocytosis	Haematocrit	Rate of infection during bacterial challenge
	♀	♂	1st set	2nd set				
009 356 316 (ICL A)	100	0	Slow	Fast	Medium	High	Medium	Low
010 036 092 (ICL B)	100	0	Slow chronic	Fast	Medium	Low	High	High
006 812 566 (ICL C)	100	0	-	-	High	High	Low	Low
OCL AxB	100	0	-	-	Higher than parents	Higher than parents	IBP (ICL B dominant)	IBP (ICL A dominant)
OCL BxC	100	0	-	-	IBP (Additive parental effects)	IBP (ICL C likely dominant)	IBP (ICL B dominant)	IBP (ICL C dominant)
OCL CxA	100	0	-	-	IBP (Additive parental effects)	IBP (ICL A likely dominant)	Higher than parents	Higher than parents
000 886 064 (ICL D)	100	0	Slow	Fast	-	-	-	-
002 041 887 (ICL E)	37.5	62.5	-	-	Low	Low	High	-
002 046 539 (ICL F)	75	25	-	-	-	-	-	-
010 805 638 (ICL G)	100	0	-	-	-	-	-	-

*Clonal lines are designated by their mitotic mothers tag number
ICL- Inbred clone; OCL- Outbred clone
IBP-intermediate between parents
- not tested

demonstrated a correlation between the level of infection and the non-specific immune parameters investigated such as the phagocytic activity of macrophages and in some cases lysozyme activity. The ICL B showed significantly higher response to *A. hydrophila* than other two lines, ICL A and ICL C. There are some reasons behind the

differential response of the clonal lines to the challenged bacteria. Fish in the ICL B showed significantly lower level of phagocytic activity of macrophages than that of the ICL A and ICL C. This lower phagocytic activity of ICL B might be involved in their higher susceptibility to *A. hydrophila* infection. In other way, fish in the ICL A and ICL C showed significantly higher levels of phagocytosis of bacteria than ICL B and as a result they had a less susceptibility to *A. hydrophila* infection. Apart from phagocytic activity of macrophages, serum lysozyme activity might play an important role to make a line more or less susceptible to challenged pathogen but it was not so clear in this study, because the ICL A and ICL B both showed similar levels of lysozyme activity but the level of disease susceptibility of ICL B was higher than that of the ICL A. On the other hand, the ICL C showed a higher level of lysozyme activity and a lower level of disease susceptibility. The haematocrit values in different clonal lines did not show any consistent relationship with other parameters. The analysis of disease susceptibility to *A. hydrophila* indicated a clear genetic difference between inbred lines. It also suggested that the homozygous alleles fixed in ICL A and ICL C might be more resistant to disease than ICL B and these alleles elicited strong immune response against the challenged pathogen.

In the case of outbred clones, the disease susceptibility levels were intermediate to that of their parents and there was a relationship between their non-specific immune response and disease susceptibility. The analysis of non-specific immune responses in OCLs showed that the parents had a genetic effect on their outbred offspring. For example, the ICL C had dominant effect in its BxC progeny. The intermediate response to bacterial challenge of the OCLs revealed that there was a definite genetic effect on disease resistance. It also suggested that by crossbreeding between a high and a low

resistant population, high resistance alleles can be transmitted in the offspring and thus more disease resistant population can be obtained.

Amin and Abdel-Kerim (1976) reported that intraperitoneal injection of bacteria was a suitable route for infecting common carp. Although *A. hydrophila* was injected intraperitoneally into the fish in this study, other routes of inducing this bacterial infection have also been described. As well as intraperitoneal injection of *A. hydrophila*, Esteve *et al.* (1993) artificially induced disease in European eels (*Anguilla anguilla*) by waterborne exposure (bath challenge). They observed that intraperitoneal injection caused acute infection, whereas infection by bath challenge progressed more slowly and produced fewer mortalities. Suthi (1991) demonstrated infection in *Puntius schwanenfeldi* and *Oreochromis niloticus* by bath challenge with *A. hydrophila* and infection was possible in both fish with and without skin abrasions. Sharifpour (1997) found that intramuscular injection of *A. hydrophila* produced ulcers on the body surface of common carp, *C. carpio* 2 days after injection, and killed 33.3% of the total fish. A similar route of infection has been reported in *O. niloticus* by Millar (1994).

The production of resistant hybrids has been reported in American catfish (*Ictalurus punctatus*) by Plumb *et al.* (1975). They produced hybrids by crossing between a resistant population and a susceptible population and the resulting hybrid fish showed fewer mortalities (9%) from viral diseases than their susceptible (29%) and resistant (13%) parents. The cumulative mortalities from this study showed that the ICL B was the most susceptible clonal line to *A. hydrophila* infection. When this line was crossed with the more resistant clonal lines, ICL A and ICL C, the resulting progenies exhibited higher resistance than that of their lower resistant parent. From these results it can be assumed that crosses between two homozygous populations more likely exhibit

significant heterozygote dominant effect and this factor may increase the disease resistance in the crossed populations.

Variations in disease resistance between populations have been found in fish. Wolf (1953) demonstrated variations in mortality between strains, ranging from 35.7 to 65.8% in brown trout and from 5.3 to 97.8% in brook trout. Silim *et al.* (1982) reported a wide variations in mortality due to IPN infection, ranging from 30.9 to 72.3% between three species of brook trout. The variations in disease resistance between these populations lead scientists to produce disease resistant strains by selective crossbreeding. Many studies on disease resistance of brown and brook trouts have been conducted in USA (Wolf, 1953; Snieszko *et al.*, 1959; Ehlinger, 1977). These studies have led to considerable increases in the level of disease resistance in fish through strain selection. Although a large amount of literature is available on this subject, most of the works were involved with natural outbred populations (Pojoga, 1972) or at best partially inbred strains (Hines *et al.*, 1974). To accurately analyse genetic variation for disease resistance in fish, it is desirable to use fully homozygous individuals. In this respect, mitotic gynogenesis and other approaches adopted in this thesis may be useful for such analysis.

Although clonal lines were used in this study to analyse the genetic influences on immune response, they can also be used in many other research purposes:

i) clones are the gynogeneticaly or androgenetically produced second generation i.e. they are usually produced from mitotic females or androgenetic males. Clonal individuals are homozygous for every gene locus and they are identical to their homozygous mother or father. Therefore, the sex ratio of clones can be used in studies on sex determining mechanism of a fish population or species.

ii) clonal lines can be used in genome mapping. The techniques of gene mapping can provide inside knowledge of gene organisation on the chromosome, gene regulation and their developmental processes. It also helps to developing marker loci which can increase the efficiency of breeding programme of commercially important fish species through marker assisted selection.

iii) since clones are completely homozygous animals, any interesting gene (e.g. disease resistance, growth regulation) and their functional expression can be examined in them.

iv) clones are free from recessive lethal and deleterious alleles and can be considered as a starting population in improving fish stocks.

v) clones are pure and identical animals, so they can be used in standardisation of bioassays such as toxicological, endocrinological and water pollution studies. For example, if the clonal individuals of a line are exposed to different concentrations of a toxicant, they will respond differently and as a result, a variable level of response to the toxicant can be obtained. These differential responses of clones can be used to standardise the toxicological study. Similarly, the levels of natural water pollution and its effect on aquatic life can be examined using clonal lines.

The analysis of immune response in tilapia during this study highlighted two points- genetic variation exists between clonal populations which results in variation in disease resistance, and the possible improvement of disease resistance in the outbred clones by crossbreeding. Since the clonal lines of tilapia are grouped by MHC haplotypes, it should be highlighted that MHC haplotypes along with other genes are

involved in disease resistance of these species. Variation in resistance between clonal lines in this study results from non-specific immune response, while MHC may control responses of T lymphocytes and their memory function. Although the present research was in part an attempt to analyse the involvement of MHC genes in immune response of tilapia, further research relating to the MHC of the tilapia needs to be performed and the following areas are suggested for further investigation.

It is very important to determine and characterise the total MHC haplotypes which exist in tilapia. The MHC haplotypes can be determined by sequencing the MHC genes using inbred or clonal (gynogenetic or androgeneic) individuals. As it is known that the number of loci varies in MHC haplotypes, functional expression of different MHC haplotypes needs to be determined. This can be partially conducted by scale or skin grafting between homozygous inbred strains. Although disease resistance of a naïve fish is non-specific, the participation of a particular MHC haplotype in disease resistance requires to be examined. This can be carried out by analysing segregation of different MHC haplotypes from clonal lines in the subsequent generations. The segregation of MHC haplotypes and their response can be examined by producing F1 and F2 generations through crossbreeding between a high and low disease resistant clonal lines and challenge them with a pathogenic bacteria or parasite.

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Appendices

Appendix 2

2.1 Modified Fish Ringers solution (MFR)

Ingredients	Original Chemical composition	Remodified chemical composition
NaCl	3.25 g	3.25 g
KCl	1.50 g	2.50 g
$NaHCO_3$	0.10 g	0.10 g
$CaCl_2, 6H_2O$	0.15 g	0.15 g
Distilled water	500 ml	500 ml
pH	8.0	8.3

Solution stored at 4 °C.

2.2 Cortland's saline

Ingredients	Amount
NaCl	1.81 g
$CaCl_2, 2H_2O$	0.04 g
NaH_2PO_4	0.09 g
$NaHCO_3$	0.25 g
$MgSO_4$	0.06 g
Glucose	0.25 g
EDTA	0.23 g

Dissolved in 250 ml distilled water.

2.3 Tris-Borate-EDTA (TBE) buffer for starch gel electrophoresis

Ingredients	Amount
Tris (0.5 M)	60.57 g
Boric acid (0.24 M)	15.00 g
EDTA (0.016 M)	5.99 g

Dissolved in distilled water to make the total volume 1000 ml and pH adjusted to 8.5. For electrode undiluted buffer was used but for gel preparation it was diluted 1:10 with distilled water.

Appendix 2 (Continued)

2.3.1 Stain

ADA (Adenosine deaminase)

Ingredients	Amount
ADA	15 mg
MTT	5 mg
PMS	1 mg
XOD (0.025 U)	4 µl
NP (0.625 U)	10 µl

Mixed with 25 ml 0.05M PO_4 buffer (pH 7.8) and then 25 ml 2% boiled agar (50-60 ^{o}C) was added.

2.3.2 Fixing solution for starch gel stain

Ingredients	Amount
Acetic acid (glacial)	200 ml
Methanol	800 ml
Distilled water	1000 ml

Mixed the ingredients thoroughly.

2.4 TBE buffer for agarose gel electrophoresis

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

Dissloved in deionized distilled water to make the total volume 1000 ml and pH adjusted to 8.3. Sterilised the buffer by autoclaving.

Appendix 3

3.1 Histological processing schedule for automatic tissue processor

50% methylated sprit	1 h
80% methylated sprit	2 hrs
100% methylated sprit	2 hrs
100% methylated sprit	2 hrs
100% methylated sprit	2 hrs
100% ethanol	2 hrs
100% ethanol	2 hrs
Chloroform	1 h
Chloroform	1 h
Molten wax	2 hrs
Molten wax	2 hrs
Molten wax	2 hrs

3.1.1 Haematoxylin-Eosin staining protocol for tilapia scale slides

Xylene	5 mins
Absolute alcohol I	2 mins
Methylated sprit	1.5 mins
Wash in tape water	1 min
Haematoxylin	5 mins
Wash in tape water	1 min
Acid alcohol	3 quick dips
Wash in tape water	1 min
Scott's tap water	1 min
Wash in tape water	1 min
Eosin	5 mins
Wash in tape water	1 min
Methylated sprit	30 sec
Absolute alcohol II	2 mins
Absolute alcohol I	1.5 mins
Xylene (Clearing)	5 mins
Xylene (Coverslip)	upto mounting
Mounting	DPX

Appendix 4

4.1 Sodium phosphate buffer (pH 5.75, 0.04M)

Ingredient	g l^{-1}
Na_2HPO_4, $2H_2O$	7.12 g
NaH_2PO_4, $2H_2O$	6.24 g

Dissolved in 1 litre of distilled water

4.2 Lysis Buffer

0.1 M citric acid
1% Tween 20
0.05% crystal violet

4.3 Phosphate Buffer Saline (PBS, pH 7.2, 0.02 M)

Ingredient	g l^{-1}
NaH_2PO_4, $2H_2O$	0.876 g
Na_2HPO_4, $2H_2O$	2.56 g
$NaCl_2$	8.77 g

Dissolved in 1 litre of distilled water

4.4 Preparation of culture media for bacteria

4.4.1 Tryptone Soya Agar (TSA)

Ingredient	g l^{-1}
Tryptone	15.0
Soya peptone	5.0
Sodium chloride	5.0
Agar	15.0

pH 7.3 ± 0.2

Appendix 4 (continued)

40 g TSA was resuspended in 1 litre of distilled water and boiled until dissolved completely. The medium was then sterilised by autoclaving at 121 °C for 15 mins.

4.4.2 Tryptone Soya Broth (TSB)

Ingredient	g l^{-1}
Pancreatic digest of casein	17.0
Papaic digest of soyabean meal	3.0
Sodium chloride	5.0
Dibasic potassium phosphate	2.5
Glucose	2.5

pH 7.3 ± 0.2

30 g of TSB was resuspended in 1 litre of distilled water and distributed into final containers. The medium was then autoclaved at 121 °C for 15 minutes.

4.4.3 Oxidative-Fermentative Medium (O-F Medium)

Ingredient	Amount
O F Basal medium	4.7 g
Agar No. 1 (0.1%)	0.5 g
1% glucose	5.0 g
Distilled water	500 ml

The ingredients were resuspended in water and heated in a microwave until dissolved. The medium was mixed thoroughly by inverting the bottle and then aliquoted 9 ml into each O-F tube using a 10 ml pipette. After aliquoting, the tubes were inverted 2-3 times to ensure that the agar did not sink to the bottom of the tube and were then

Appendix 4 (continued)

autoclaved at 121 °C for 20 mins. The medium was left for approximately 12 hrs before use.

4.5 Tests for identification of bacteria

4.5.1 Gram's Stain for bacteria in smears

The morphology of bacteria is difficult to observe in wet and unstained preparations. Usually thin films of organisms were prepared and stained in order to examine them. The Gram's stain is a differential stain and is the most important and widely used technique in bacteriology, as it divides nearly all bacteria into one of two categories.

Gram positive- resist decolourisation by ethanol or acetone and stain blue/purple

Gram negative- are decolourised by ethanol or acetone and are stained red/pink.

The difference in the colour reaction is due to the different chemical composition of the cell wall between bacteria.

Method

1. A loopful sterile saline was placed on a clean microscope slide
2. A minute quantity of bacterial culture was removed from an agar plate using a sterile loop and emulsified in the liquid on the slide and spread evenly
3. The slide was allowed to air dry
4. The slide was held with the film upwards using forceps and slowly passed through a Bunsen flame three times to fix the film
5. The slide was allowed to cool and placed in a staining dish

Appendix 4 (continued)

6. The slide was immersed in crystal violet solution and left for approximately 1 min
7. The residual stain was washed off with water, then the slide was immersed in iodine and left for approximately 1 min
8. The slide was then placed in alcohol/acetone mixture and mixed gently for approximately 10 seconds
9. The slide was washed thoroughly with water
10. The slide was placed in safranine solution for approximately 2 minutes
11. The slide was washed thoroughly with water, then dried and cleaned the bottom
12. Microscopically the stained slide was examined using first x40 objective and then x100 objective under oil immersion

4.5.2 Motility test

This test demonstrates whether a bacteria is capable of independent movement, i.e. is motile. Many species of bacteria are capable of motility by the movement of external appendages called flagella. This motility can be observed directly under the microscope using a suspension of living bacteria in a "hanging drop slide". A drop of bacterial suspension is hung from the underside of a coverslip and mounted, using soft paraffin, on a microscope slide. Direct observation of the slide under the x40 objective lens of a microscope will then reveal whether the bacteria are motile.

All bacteria in suspension exhibit movement which is quite random and non-directional, whereas only some bacteria exhibit true motility which is non-random and directional.

Appendix 4 (continued)

Method

1. Vaseline was placed on the four corners of a coverslip

2. The coverslip was placed, vaseline up, on the bench

3. A loopful sterile saline was placed on the coverslip. The loop was re-sterilised and a very small amount of bacterial growth from an agar plate was picked up by it and gently emulsified in the saline

4. A microscope slide was gently placed onto the vaseline mounds without allowing the slide to touch the drop of culture

5. The slide was then quickly, but gently inverted so that the drop was hanging from the coverslip

6. The slide was carefully placed onto the microscope and the x40 objective lens was rack down until it was just touching the coverslip

7. The objective lens was slowly racked up until the image was formed

8. Finally the bacterial movement was examined

4.5.3 Oxidase test

This test demonstrates whether a bacterium possesses certain oxidase enzymes that are involved in electron transfer from electron donors. If the redox dye tetramethyl-p-phenylenediamine is used as the electron acceptor, this will be reduced and showed a deep blue colour.

Growth from an agar culture of the organism to be tested is smeared onto a pre-prepared freeze-dried reagent strip (Oxidase strip) and the colour of the smear is noted after 30 seconds. It is important to note that bacterial culture must be smeared using a

Appendix 4 (continued)

platinum wire as other bacteriological wires may contain traces of iron which could catalyse the reaction and give a false positive result.

Method

1. An "oxidase strip" was placed into a clean Petri dish
2. A platinum wire was sterilised and allowed to cool
3. A heavy inoculum of pure growth from a culture plate was picked up using the tip of the wire
4. The inoculum was smeared over the area of filter paper containing the "oxidase reagent"
5. The wire was re-sterilised
6. The smear was observed for up to 30 seconds and noted any colour change

Result

A deep blue or purple colour developing within 30 seconds indicates oxidation of the reagent and a positive result.

4.5.4 O-F test (Hugh and Liefson)

This test demonstrates whether bacteria can break down glucose aerobically (by oxidation) or anaerobically (by fermentation).

A culture of the organism is inoculated into freshly prepared tubes of O-F medium by a single stab with a straight wire. One tube is incubated in the presence of air (open, aerobic tube); the other is covered with a thick layer of liquid paraffin to

Appendix 4 (continued)

exclude air (closed, anaerobic tube). The medium contains bromothymol blue pH indicator to indicate the formation of acid from the breakdown of glucose.

Method

1. A straight wire was sterilised and allowed to cool
2. An inoculum of pure growth from a culture plate was picked up using the tip of the straight wire
3. A single stab was made into the agar of one tube using the inoculated straight wire
4. The second O-F tube was inoculated in an identical manner
5. The agar in one tube was aseptically covered with a 5-10 mm layer of liquid paraffin
6. Both the tubes were incubated at 20 ^{o}C for overnight

After suitable incubation the results were interpreted as follows :

Open tube	**Closed tube**	**Result**
Green	Green	No reaction on glucose
Blue at top	Green	Alkaline reaction
Yellow	Green	Oxidative
Yellow	Yellow	Fermentative

Appendix 4 (continued)

4.5.5 API 20E Microbial Identification Kit

The API 20E™ system is a standardised miniaturised version of conventional procedures for the identification of certain Gram-negative bacteria. It is a microtube system enabling 23 standard biochemical tests to be carried out on a bacterial culture. A suspension of the culture under test is prepared and inoculated into small plastic cups containing dehydrated media. After suitable inoculation, results are obtained by direct reading and in some cases after addition of reagent.

Identification of the organism to be tested may be possible by comparing the results obtained with known results for certain fish pathogens.

Preparations

Preparation of strip

1. A small volume of water was dispensed into the honeycombed wells of the incubation tray using a plastic squeeze bottle. It provides a humid atmosphere during incubation
2. The reference of the test organism was recorded on the elongated tab of the tray
3. API strip was removed from the sealed envelope and placed in the tray

Preparation of bacterial suspension

1. A bacteriological loop was sterilised and allowed to cool
2. A single well-isolated colony of bacteria from a pure culture was picked up using the sterile loop

Appendix 4 (continued)

3. 5 ml of sterile saline was taken in a sterile bottle and then inoculated the culture in the saline by gentle rubbing from the loop on the inner surface of the bottle. Unnecessary vibration of the loop was avoided

4. The loop was re-sterilised

5. The cap of the bottle was placed tightly and then shaken gently to resuspend the culture in the saline

Method

Inoculation of strip

1. A quantity of the culture was pipetted up using a sterile plastic pipette

2. The API incubation tray was tilted and carefully filled the tube section of the microtubes by placing the pipette tip against the side of the cupule

3. The cupule section of the CIT, VP and GEL tubes were filled up with the bacterial suspension

4. After inoculation, the cupule section of the ADH, LDC, ODC, H_2S and URE tubes were filled completely with sterile liquid paraffin using a sterile pipette to create anaerobiosis

5. The lid of the strip was replaced and then incubated at 20-22 ^{o}C for 2 days

Reading the results

After examining the strip, all reactions were recorded in Table 2 and the results obtained were compared with the "Summary of Results" in Table 1. For TDA, VP and IND tubes particular reagents were added which are as follows :

Appendix 4 (continued)

1. TDA test- 1 drop of TDA reagent was added to the TDA tube. A dark brown colour indicated a positive reaction.

2. VP test- 1 drop of VP1 and VP2 reagent were added to the VP tube and waited for at least 10 minutes. A bright pink or red colour indicated a positive reaction.

3. IND test- 1 drop of IND reagent was added to the IND tube. The reaction took place immediately and a pink colour developed within 2 minutes in the whole cupule which indicated a positive reaction.

Appendix 4 (continued)

Table 1 API 20E "Summary of Results"

Test	Substrate	Reaction/enzyme	Negative	Positive
ONPG	Ortho-nitrophenyl galactosidase	beta-galactosidase	Colourless	Yellow
ADH	Arginine	arginine dihydrolase	Yellow	Red/Orange
LDC	Lysine	lysine decarboxylase	Yellow	Orange
ODC	Ornithine	ornithine decarboxylase	Yellow	Red
CIT	Sodium Citrate	citrate utilisation	Yellow	Blue/Green
H_2S	Sodium Thiosulphate	H_2S production	Colourless	Black Deposit
URE	Urea	urease	Yellow	Red/Orange
TDA	Tryptophane	tryptophane deaminase	Yellow	Dark Brown
IND	Tryptophane	indole production	Yellow Ring	Red Ring in 2 minutes
VP	Sodium Pyruvate	acetoin production	Colourless	Pink/Red in 10 minutes
GEL	Gelatin	gelatinase	No Black Diffusion	Black Diffusion
GLU	Glucose	fermentation/oxidation	Blue/Green	Yellow
MAN	Mannitol	fermentation/oxidation	Blue/Green	Yellow
INO	Inositol	fermentation/oxidation	Blue/Green	Yellow
SOR	Sorbitol	fermentation/oxidation	Blue/Green	Yellow
RHA	Rhamnose	fermentation/oxidation	Blue/Green	Yellow
SAC	Sucrose	fermentation/oxidation	Blue/Green	Yellow
MEL	Melibiose	fermentation/oxidation	Blue/Green	Yellow
AMY	Amygdalin	fermentation/oxidation	Blue/Green	Yellow
ARA	Arabinose	fermentation/oxidation	Blue/Green	Yellow

Appendix 4 (continued)

Table 2 Characteristics of *Aeromonas hydrophila* and *Areomonas sorbia* determined by conventional methods and the API 20E system

Characteristics	*Aeromonas hydrophila*	*Aeromonas sorbia*
Rods in singles and pairs	+	+
Motility	+	+
Oxidase	+	+
O/F	+	+
Growth in TSB at 37 °C	+	+
ONPG	+	+
ADH	+	+
LDC	+	+
ODC	–	–
CIT	+	+
H_2S	–	–
URE	–	–
TDA	+	+
IND	+	+
VP	+	+
GEL	+	+
GLU	+	+
MAN	+	+
INO	–	–
SOR	–	–
RHA	–	–
SAC	+	+
MEL	–	–
AMY	+	+
ARA	+	+

Appendix 4 (continued)

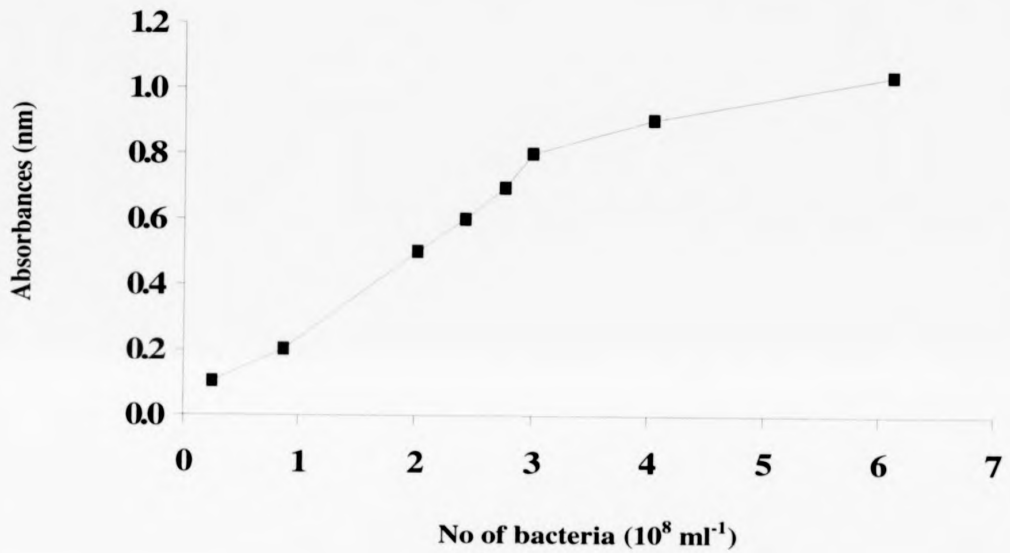

Figure 1 Concentration of *Aeromonas hydrophila*, strain T4 (bacteria ml^{-1}) at an absorbance of 610 nm