A Comparison of Immune Responses and Disease Resistance in Clonal Lines of Nile Tilapia *Oreochromis niloticus* L.

A thesis submitted for the Degree of Doctor of Philosophy

By

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To my husband, best friend and soul mate
Dr Tariq Ezaz
Whose endless love, support and sacrifice have made it possible to complete this thesis

And

To my parents
Saleha Khatoon
Syed Ahmed
For their love, moral support and blessings

DECLARATION

I declare that this thesis has been composed by myself based on my own research. It has neither been submitted nor accepted for any other degree. All information from other sources has been properly acknowledged.

Sayema Sayeed

ABSTRACT

Five clonal lines of Nile tilapia *Oreochromis niloticus* were produced and propagated. Three second generation clonal lines (clonal lines A, B and C) were produced from females from the respective clonal lines by meiotic gynogenesis followed by sib mating between female and sex reversed neomales from the same clonal line. Clonal lines D and E were first generation clones produced by meiotic gynogenesis from an XX mitotic gynogenetic daughter of an XY neofemale. Survival rate at both pigmentation and yolk sac absorption stages were poor in the meiotic gynogenetics. No males were observed in any of the clonal lines.

Microsatellite loci in multiplex reactions were used for parentage analysis of the clonal lines. Analysis of data revealed that clonal individuals from all clonal lines inherited only maternal alleles.

The specific immune response were studied in Clonal lines A and B, an outbred clonal line OBC (AXB) and an unrelated control group of Nile tilapia following immunisation with SRBC, DNP-KLH and TNP-LPS. The specific immune response of clonal line A was significantly higher than clonal line B. The OBC (AXB) line had an intermediate response between clonal lines A and B, which was significantly higher than clonal line A and significantly lower than clonal line B. The response of URC was lower than clonal line A but the difference was not statistically significant.

The experimental groups of fish had similar patterns of specific immune response after being vaccinated with heat killed *Aeromonas hydrophila* T4. The experimental challenge with *A. hydrophila* T4 revealed an inverse relationship between antibody production and susceptibility to disease. Clonal line A had significantly lower mortality

than clonal line B. Mortality in OBC (AXB) was significantly higher than clonal line A and significantly lower than clonal line B.

Lymphocytes isolated from peripheral blood, spleen and head kidney of the experimental groups of fish were stimulated with T-cell mitogen Con A and B-cell mitogen LPS. No correlation was found between polyclonal activation of lymphocytes of healthy fish and specific immune response or disease resistance in the same clonal line.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
APC	antigen presenting cells
APS	ammonium persulphate
ASC	antibody secreting cell
BSA	bovine serum albumin
CCV	channel catfish virus
CFU	colony forming unit
cm	centimetre
Con A	Concanavalin A
CPM	counts per minute
DAB	3-3-diaminobenzidine tetrahydrochloride
DNP-BSA	dinitrophenyl-bovine serum albumin
DNP-HSA	dinitrophenyl-human serum albumin
DNP-KLH	dinitrophenyl-keyhole limpet haemocyanin
dNTP	2'-deoxynucleoside 5'triphosphate
DPM	disintegration per minute
EDTA	ethylenediamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
FAM	fluorescein phosphoramidite NHS ester
ELISPOT	enzyme linked immunospot assay
FBS	foetal bovine serum
g	Gram/gravitational force
HEX	Hexachloro- fluorescein phosphoramidite NHS ester
HRP	horseradish peroxidase
HSW	high salt wash buffer
IAM	infinite allele mutation
IgG	immunoglobulin G
IgM	immunoglobulin M
IHN	infectious haematopoetic necrosis

IL-1	interleukin-1
i.p.	intra-peritoneal
IPN	infectious pancreatic necrosis
kb	kilobase
kDa	kilo Dalton
kg	kilogram
l	litre
LA	lymphocyte activation
LPS	lilopolysaccharides
M	molar
MAb	monoclonal antibody
MAS	motile Aeromonas septicemia
MFR	modified fish Ringers
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
nm	nanometer
OBC	outbred clonal line
OD	optical density
OMP	outer membrane protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFC	plaque forming cell
PIT	passive integrated transponder
QTL	quantitative trait loci
RLP	relative level of protection
S.I.	stimulation index
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electropheresis

sec	second
SMM	stepwise mutation model
SRBC	sheep red blood cells
TAE	tris acetic acid EDTA
Taq	*Thermas aquaticus*
TBS	tris buffered saline
TCR	T cell receptor
TD	thymus-dependent
TE	tris EDTA
TEMED	N,N,N'N'-tetra methylethylenediamine
TEN	tris EDTA sodium chloride
TET	Tetrachloro fluorescein phosphoramidite NHS ester
TI	thymus-independent
TMB	tetramethyl benzidine dihydrochloride
TNP-LPS	trinitrophenyl-lipopolysaccharide
TSA	tryptone soya agar
TSB	tryptone soya broth
TTBS	tris buffered saline with 0.005% v/v Tween-20
UNH	university of New Hampshire
URC	unrelated control
UV	ultraviolet
V	volt
V.	version
VHS	viral haemorrhagic septicaemia
VNTR	variable number of tandem repeats
v/v	volume/volume
w/v	weight/volume
$\chi 2$	chi square
°C	degree centigrade
µCi	micro curie
µg	micro gram

µl	micro litre
µM	micro molar
µW	micro watt

LIST OF FISH SPECIES

Common English name	Scientific name
African catfish	*Clarias gariepinus*
Air breathing catfish	*Clarius batrachus*
Amago salmon	*Oncorhynchus rhodurus*
Amazon molly	*Poecilia formosa*
American catfish	*Ictalurus furcatus*
Arctic char	*Salvelinus alpinus*
Atlantic menhaden	*Brevoortia tyranus*
Atlantic salmon	*Salmo salar*
Ayu	*Plecoglossus altivelis*
Blue gourami	*Trichogaster trichopterus*
Blue tilapia	*Oreochromis aureus*
Bluegill	*Lepomis macrochirus*
Bream	*Abramis brama*
Brook trout	*Salvelinus fontinalis*
Brown trout	*Salmo trutta*
Channel catfish	*Ictalurus punctatus*
Chinook salmon	*Oncorhynchus tshawytscha*
Coho salmon	*Oncorhynchus kisutch*
Common carp	*Cyprinus carpio*
Cutthroat trout	*Oncorhynchus clarki clarki*
Cyprinid loach	*Misgurnus anguillicaudatus*
Dab	*Limanda limanda*
Damselfish	*Pomacentrus partitus*
European eel	*Anguilla anguilla*
European seabass	*Dicentrarchus labrax*
Goldfish / Crucian carp	*Carassius auratus*
Grass carp	*Ctenopharyngodon idella*
Hirame	*Paralichthys olivaceus*
Indian major carp	*Labeo rohita*

Common English name	Scientific name
Japanese flounder	*Paralichthys olivaceus*
Medaka	*Oryzias latipes*
Milk fish	*Chanos chanos*
Mozambique tilapia	*Oreochromis mossambicus*
Nile tilapia	*Oreochromis niloticus*
Plaice	*Pleuronectes platessa*
Rainbow trout	*Oncorhynchus mykiss*
Red seabream	*Pagrus major*
Roach	*Rutilus rutilus*
Silver barb	*Barbodes gonionotus*
Sockeye salmon	*Oncorhynchus nerka*
Southern platyfish	*Xiphophorus maculatus*
Spotted gar	*Lepisosteus platyrhinchus*
Striped bass	*Morone saxxatilis*
Three spotted tilapia	*Oreochromis andersonii*
White bass	*Morone chrysops*
Zebrafish	*Danio rerio*

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Aquaculture is the fastest growing food production sector in the world and cultured finfish and shellfish accounted for over 25 percent world aquatic animal production (FAO, 2002). According to the intensity of aquaculture, it can be categorised into extensive, semi-intensive and intensive. Semi-intensive and intensive aquacultures are being practised more widely because of the recent developments in culture techniques. Huge numbers of hatcheries have been established worldwide to maintain a steady supply of seed to the farmers. Due to the continuous depletion in the capture fisheries because of poor management, culture fisheries are facing immense pressure to meet the increasing demand. However, poor management in hatcheries is a constraint to increased aquaculture production. Deterioration of the culture environment will lead to problems with disease, not only because of the increased number of animals in a limited and confined space, but also because of the influence of poor environmental conditions such as water quality on the fish immune system. As a result there will be an increased and continued negative effect of disease on production (Shoemaker *et al.*, 2001). In recent years, the necessity of genetic management of culture species has come to light, however, improvements have been limited to only a few species such as salmon, carp and tilapia (Kinghorn, 1983; Eknath *et al.*, 1993).

In intensive culture, fish are usually kept at relatively high densities which can result in stress and eventually disease. Disease can cause serious problems in aquaculture, sometimes destroying the whole production cycle. To control or reduce the risk of diseases in intensive aquaculture usually four basic steps are followed: (i) treatment with drugs, (ii) good husbandry i.e. tank cleaning, water aeration, disinfection of egg/fry

before stocking; killing of diseased fish and replacing stocks after disinfection (iii) vaccination and (iv) establishment of disease resistance by selection and genetic manipulation. Although the first three measures have been applied frequently (Hayes, 1984; de Kinkelin *et al.*, 1984; Lamers, 1985), only little information is available on the fourth. The concept of establishing genetic resistance is receiving a lot of attention because of the problems associated with treatments with drugs and availability and cost of vaccination (Rijkers *et al.*, 1980b; Grondel and Boesten, 1982; Erdal and Reitan, 1992).

1.2. Immunity and disease resistance in fish

The state of being immune is defined as the inherited ability to resist infection. Immunity is the result of the recognition of nonself or a foreign agent, with the subsequent response and memory in vertebrate animals. The response includes expansion of cells for the immune response, expression of the cells and molecules (e.g. antibody), and finally, the co-ordination of the response by regulatory substances. Disease resistance is the non-specific or natural defence non-specific mechanism of an organism against any foreign agent (Shoemaker *et al.*, 2001). Natural defence mechanism and specific (acquired) inmunity co-ordinate to protect fish from pathogen. Fig. 1.1 is a schematic representation of the result of the response to a pathogen by fish.

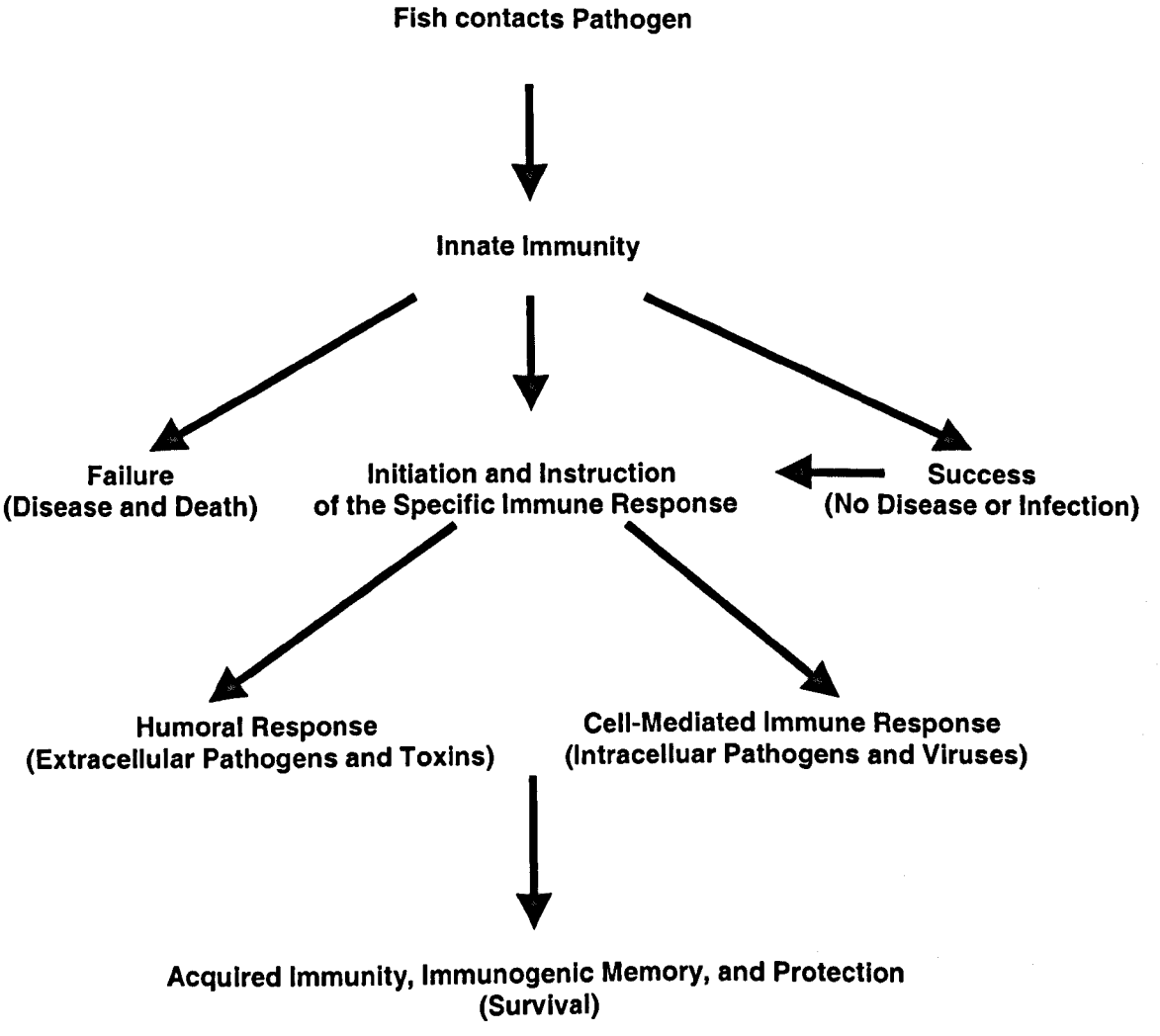


Fig. 1.1. Schematic representation of the response of a fish following an encounter with a pathogen (after Shoemaker *et al.*, 2001).

In order to fulfil the fundamental Darwinian tenet of the survival of the fittest, multicellular organisms must possess some means of maintaining their integrity in the face of pathogenic onslaught. In the most primitive organisms this is achieved by the innate immune system, a rapid, non-specific mechanism, which is ancient and highly conserved. In 98.6% of multicellular organisms only this system is present and it serves them well. The other 1.4% of multicellular organisms possess two branches to their immune system, a specific immune response and a non-specific immune response. The

specific immune system is also referred to as adaptive, acquired, anticipatory and most recently combinatorial reflecting the genetic mechanisms involved. It has been hypothesised that evolution of the specific immune system occurred suddenly, at the level of jawed vertebrates around 450 million years ago (Schluter *et al.*, 1999). The evolution of this system introduced antigen specificity and enhanced resistance to re-infection due to immunological memory.

There is highly complex interplay between non-specific and specific immune mechanisms. In fish, the primary line of defence is the non-specific or natural immune response. If antigen successfully penetrate the first line of defence, "danger signals" arising from tissue damage and cell stress triggers activation of the innate system, which turns on the co-stimulators essential for activation of the specific immune system. In mammals, the specific immune system is at its most sophisticated and it is the most highly characterised. As such, it provides a framework within which the components of fish immune system can be contrasted and compared. Although the development of piscine immune system is different from mammalian system reflecting differences in physiology, there are many common features of form and function (Ellis, 1982; Watts *et al.*, 2001).

1.2.1. Lymphoid organs and cells

The main lymphoid organs in adult teleosts are the thymus, head and trunk kidney, spleen and intestine (Fange, 1982; Rombout *et al.*, 1986, 1993). Some lymphocytes are also found in skin and gills. Bone marrow, bursa and lymph nodes are lacking. Most observations support the idea that the spleen of bony fish is an erythropoietic and

secondary lymphoid organ, whereas the thymus is more of a primary lymphoid organ. The kidney is a mixture of both. However, there are indications that the head kidney has more of the characteristics of a primary organ than the trunk kidney (Van Muiswinkel, 1992).

Cells which participate in the specific immune response in teleosts have been classified following the mammalian convention of nomenclature and the cell types are comparable (Clem *et al.*, 1981; Manning, 1994). Based on functional studies (Stolen and Makela, 1975; Miller *et al.*, 1985) and on reaction with monoclonal antibodies (DeLuca *et al.*, 1983; Secombes *et al.*, 1983; Miller *et al.*, 1987), distinct B and T lymphocyte populations have been identified (Kaattari, 1992). Also the role of macrophages as antigen presenting cells (APC) has been well documented (Secombes, 1996). Graft rejection and delayed type hypersensitivity reactions have been demonstrated, suggesting specific cell-mediated immunity via cytotoxic T-lymphocyte activity (Nakanishi, 1999).

1.2.2. The non-specific immune system

The non-specific immune system is composed of cell mediated and humoral immune responses. Components of the cellular system such as macrophages, granulocytes, neutrophils, and natural killer cells combine with the soluble humoral factors such as lysozyme, complement, interferon, cytokines, C-reactive protein, transferrin and lectin to provide fish with innate protection. The skin and mucous of fish act as natural barriers to foreign substances and disease agents.

1.2.3. The specific immune system

The specific immune system of fish comprises cell mediated and humoral (antibody production) immune responses. Mitchinson (1953) first reported that lymphocytes are responsible for specific cell mediated immunity. The specific immune response involves two major groups of cells: lymphocytes and macrophages or antigen presenting cells (APC). Though macrophages are an important component of non-specific cell mediated immunity, they also play a vital role in specific immune response. B lymphocytes express immunoglobulins (Ig) on their surface and act as the receptor of antigen whereas the T lymphocytes are characterised by a different type of antigen specific receptor, the T cell receptor (TCR).

1.2.3.1. Activation of B cell

From the point of view of B lymphocyte activation, antigenic structure can be divided into two forms, thymus-dependent (TD, proteins) and thymus-independent (TI, polysaccharide). The induction of antibody responses to TI antigen requires the auxiliary assistance of monocytes or macrophages (Clem *et al.*, 1985; Vallejo *et al.*, 1990, 1992; Kaattari, 1992). TI antigens trigger a receptive state in the antigen-specific B lymphocyte while simultaneously inducing the macrophage to produce interleukin-1 (IL-1). One aspect of this receptor state of B lymphocyte is the ability to receive IL-1 factor. This IL-1 factor is critical in differentiation of B lymphocyte into an antibody secreting plasma cell and not simply the enhancement of cellular proliferation (Kattaari and Piganelli, 1996). TD antigens require a sophisticated or complex co-operation between macrophages, T lymphocytes (T-helper cell) to activate B lymphocytes.

Macrophages trap and process exogenous protein molecules or particles (antigens) and present the processed relatively small antigenic determinants in association with major histocompatibility complex (MHC) class II molecules to T helper cells (Vallejo *et al.*, 1990, 1992). T-helper cells become activated by its interaction with antigenic determinanats via the T cell receptor (TCR) present on its cell membrane and factors such as IL-1 secreted by macrophages. These activated T-helper cells stimulate B lymphocytes by secretion of different factors (e.g. IL-2) and present the antigen to B lymphocytes. B lymphocytes either develop into long lived memory B cells or short lived plasma cells. These plasma cells secrete huge amount of specific antibodies (immunoglobulins) which bind or kill invading micro-organisms showing the corresponding determinants (Fig. 1.2) (Van Muiswinkel, 1992, 1995).

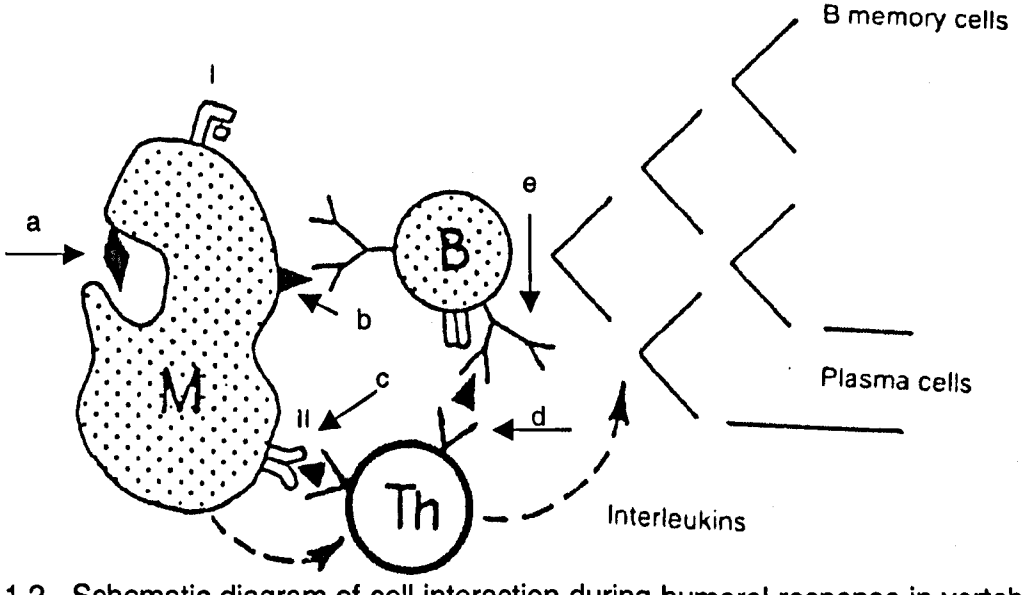


Fig. 1.2. Schematic diagram of cell interaction during humoral response in vertebrates. a: antigen; b:small antigenic determinanats; c: MHC class II molecules: d: TCR; e: antibody; M:macrophage; Th: T helper cell; B: B lymphocyte (after Van Muiswinkel, 1995).

The presence of a specific humoral response which requires B and T-helper lymphocytes and antigen presenting cell collaboration has been observed in all teleost species studied (Kaattari, 1992; Manning, 1994). This is manifested by the production of antibodies against virus, bacteria, helminths and protozoa (Woo, 1992; Secombes and Chappell, 1996; Ellis, 1999; Lorenzen and Lapatra, 1999). Diagnostically the antibody response has been used to detect subclinical infection and the carrier state of a range of diseases although this approach is used less often than in terrestrial livestock. This is partly due to response being variable depending on nature of antigen and the route of exposure (Van Muiswinkel, 1992).

1.2.3.2. Fish antibody structure and function

Antibodies are among the most structurally complex biological molecules. The need for such complexity is obvious for within their structure lie the ability to bind a virtual universe of pathogens and ensure their destruction and removal. Initial characterisation of fish antibody structure isolated from serum has led to the general supposition that fish immunoglobulin (Ig) is comparable to mammalian IgM isotype (Kaattari and Piganelli, 1996).

In the teleost, this molecule appears primarily as a tetramer, composed of four monomeric subunits but some possess a monomer (Lobb and Clem, 1981a; Bang *et al.*, 1996; Zipfel, *et al.*, 1996) and a dimer has also been observed in secretions (Lobb and Clem, 1981b). As in mammals, each subunit contains two "heavy" (H) or larger (~71-80 kilo Dalton, kDa) protein chains and two "light" (L) or smaller (~22-30 kDa) chains. Individual H and L chain form pairs which are always held together by disulfide bonds.

Pairs of H and L chains, also joined by disulfide bonds between the two H chains, form the basic monomer unit. In most teleost IgM, the J chain, which links monomers in mammals is absent and noncovalent bonding is a frequent feature of the association of H:L chain units and monomers to form a complete tetramer (Wilson and Warr, 1992).

An antibody molecule can be anatomically divided into two regions: the antigen binding amino terminus (Fab) and the carboxy terminal effector region (Fc). Each antigen binding site (Fab) is constructed from amino terminal portions of each heavy and light chain yielding eight binding sites per antibody molecule (Kaattari and Piganelli, 1996).

Each B cell and its clonal progeny produce a unique antibody molecule. The assembly of antibody molecules via disulfide cross-linking appears to be a means by which structural diversity is generated. A great diversity of antigen binding sites are necessary to accommodate the innumerable number of possible antigenic structures associated with pathogens (Kaattari and Piganelli, 1996).

The most direct effect of the interaction of an antibody with the antigen is the simple physical act of blocking a critical function of the antigen by binding to a receptor, enzymatic active site or a toxigenic determinant. Molecular antigens can also be neutralised, such as the role of antitoxins in neutralising toxins. Simple masking of the toxigenic determinant with an antibody prevents induction of the toxigenic activity, often an enzyme function such as proteolysis or lipolysis. Another consequence of the interaction of an antibody molecule with an antigen is the creation of a macromolecular antibody-antigen complex. When this process of cross-linking occurs with optimal concentrations of antigens, it agglutinates forming a large lattice of antigen and

antibody. Such complexes are then phagocytosed by macrophages. The antibodies present within the complex also facilitates the activation of complement or attach to the Fc receptors of the phagocytes, allowing these complexes to be scavenged more effectively. If the concentrations of antibodies and antigens are too great this same process can lead to pathological consequences (Kaattari and Piganelli, 1996).

Opsonisation refers to the process by which either antibody or another immunologically related molecule promotes phagocytosis of antigen by phagocytes. This is most specifically used with respect to the coating of bacteria, fungi and parasites with specific antibodies which leads to interaction and often phagocytosis of the target (Honda *et al.*, 1986; Whyte *et al.*, 1990).

Although knowledge of the complement system of fish is not complete, it is believed that fish possess a complement system that functions in concert with antibodies as observed in mammals. Activation of complement with antibody represents an essential antibody effector mechanism. The role of antibody in complement activation can be thought of most simply as a mechanism to focus this potent effector function against pathogenic targets. The binding of an antibody to an antigen results in a conformational change of the carboxy- terminal effector region (Fc) of the antibody, which permits the binding and activation of the first component of the complement system. The binding of this component results in conformational changes, which in turn facilitates the binding of other complement components. In this manner, proteolytic enzymes are produced which activate other complement components in a linear fashion, the products of which can activate other specific proenzymes. As activation is a process of enzymatic cleavage, one initial step is tremendously amplified by subsequent steps, thus resulting

in what is termed the complement cascade. At each step of this process proteolytic products are produced that can opsonise and lyse pathogens (Kaattari and Piganelli, 1996; for review see Holland and Lambris, 2002).

1.2.3.3. Memory

Memory has always been considered a hallmark of the specific immune response. However, immunological memory in fish has been defined primarily by employing paradigms based on mammalian response. These paradigms specifically emphasise a variety of differential responses during mammalian response to protein antigens. Differences between piscine and mammalian specific humoral immunity relate to memory, affinity maturation and isotype switching aspects of secondary response and to antibody heterogeneity. In comparison to mammals, the memory response is modest (Manning, 1994) and antibody heterogeneity is low (DuPasquier, 1982). It therefore has been argued that development of memory is a recently evolved characteristic (Kattarri and Piganelli, 1996). An alternative viewpoint is that the memory response is simply a differentiated response to a secondary exposure to a specific antigen (Kaattari, 1994). Memory can also be characterised by the development of an increased sensitivity to antigen and enhanced secondary response observed in primed fish is exclusively due to generation of an enlarged precursor pool and the amount of clonal proliferation induced in native and primed fish is identical (Arkoosh and Kaattari, 1991).

Affinity maturation, which in mammals occurs in the germinal centres of the spleen was thought to be absent in teleosts because of their lack of these structures (Nahm *et al.*, 1992). However, somatic mutation which leads to affinity maturation occurs in

elasmobranchs (Hinds-Frey *et al.*, 1993) and a small increase in antibody affinity has been detected in rainbow trout (reviewed by Watts *et al.*, 2001). As most teleosts produce only IgM, no class switch is possible but as these molecules are functionally quite similar to mammalian IgM, they possess a high functional affinity or avidity (Kaattari, 1994). Lack of diversity of antibody class is not a functional impediment for teleosts and can be compensated by different heavy (H) chain serum and secretory isotypes and low molecular weight IgM forms. Isotypes of IgM based on heavy and light chain heterogeneity has been described in poikilothermic vertebrates (Lobb and Olson, 1988; Pilstrom *et al.*, 1998). In channel catfish *Ictalurus punctatus*, certain H and L isotypes predominate early in the immune response (Lobb and Olson, 1988; Klesius, 1992) and in Japanese flounder *Paralichthys olivaceus* a shift to low moleular weight IgM was observed (Bang *et al.*, 1996). It has been suggested that non-covalent bonding of the IgM tetramer may introduce varying degrees of flexibility resulting in an enhanced ability to bind epitopes (Kaattari *et al.*, 1999).

1.2.4. Histocompatibility

The identification of genes involved in the regulation of defence mechanisms might provide important clues to understand and perhaps to improve disease resistance in fish. Studies with several mammalian species has shown that products of a particular set of genes, the major histocompatibility complex (MHC) play a key role in the regulation of immune response (Klein, 1986). The classical MHC class I and class II genes, as identified in warm blooded vertebrates, such as chickens, primates and rodents, have so far been found in close linkage. This close linkage has given support to the concept that

these genes are always located in a single chromosomal region known as the major histocompatibility complex. The MHC has been shown to contain apart from class I and class II, other genes known as class III genes. In addition some mammalian MHCs contain numerous so called non-classical MHC genes. Most of those genes are class I like in nature and the consensus is that those non classical class I genes, with their limited expression pattern serve a special function in presenting particular antigens (Shawar *et al.*, 1994). The class I genes are expressed in all nucleated cells whereas class II genes are more or less restricted to cells of the immune system i.e. lymphocytes and macrophages. These molecules play an important role in the development of T cell repertoire (self-tolerance) and antigen presentation. As a general rule, in mammals, non-MHC genes appear to regulate the early phase of immune response to pathogens, whereas the later phase of elimination often depends on the generation of MHC restricted T cell immunity.

There is strong evidence that MHC genes exist in all vertebrates including fish (Kaastrup *et al.*, 1989; Hashimoto *et al.*, 1990). The extensive polymorphism of MHC molecules, observed in vertebrate species including fish (Grimholt *et al.*, 1994; Dixon *et al.*, 1995; Malaga-Trillo *et al.*, 1998) is most likely favoured by differences between allelic MHC molecules in the efficacy to present antigenic peptides derived from pathogens. Therefore, MHC genes are obvious candidate genes for disease resistance (Van Muiswinkel *et al.*, 1999). In the homoiothermic vertebrates, like birds and mammals, an association has been established between MHC alleles and susceptibility to specific diseases (De Vries *et al.*, 1979; Svejgaard *et al.*, 1982). The increasing knowledge of MHC in fish is important for our ideas about regulation of immune

response in fish. Genetic differences in disease resistance in fish is well documented (Chevassus and Dorson, 1990; Houghton, *et al*, 1991; Wiegertjes *et al.*, 1996a). It will be possible to generate well defined markers for disease resistance by typing of MHC genes in fish (Van Muiswinkel *et al.*, 1999; Malaga-Trillo *et al.*, 1998).

Recently, the dogma of the MHC being a single chromosomal region has been challenged by investigation into the segregation of MHC genes in teleost fish. In the zebrafish *Danio rerio*, class I and class II genes are located in different linkage groups (Bingulac-Popovic, 1997). Independent segregation of class I and class II genes and co-segregation of class II *A* and *B* is reported in Atlantic salmon *Salmo salar.* Several classical class I loci segregate independently from each other. The absence of linkage between the different classes of major histocompatibility (MHC) genes allows for an independent segregation of immunological traits in fish, either associated with class I, cytotoxicity in response to virus infection or class II, humoral response to pathogenic bacteria (Gjedrem and Gjoen, 1995; Van Muiswinkel *et al.*, 1999).

1.2.5. Factors affecting the fish immune system

1.2.5.1. Age

In addition to any maternally derived immune protection, newly hatched fry possess a variety of non-specific defence mechanisms that provide them with protection prior to specific immunity becoming fully mature. This non-specific immunity is vitally important during the first few weeks of life when it is possibly the only as well as the first line of defence. However, it contributes to defence throughout the life cycle, as

being relatively temperature independent, it comes into play at times when the specific immune system is suppressed by low environmental temperatures (Tatner, 1986, 1996).

Tatner and Manning (1983) reported that cell mediated specific immune response was functional in rainbow trout fry as early as 14 days post hatch at 14°C and by 26 days post hatch the response was as vigorous as that seen in adult fish. In common carp *Cyprinus carpio*, specific cell mediated immunity was functional by 16 days post-hatch (Botham and Manning, 1981) and in Mozambique tilapia *Oreochromis mossambicus* by 1.5 months post-hatch (Sailendri, 1973: cited by Tatner, 1996) while the rosy barb *Puntius conchonius* was fully immunocompetent with respect to cell mediated specific immunity by six months of age when kept at 24°C (Rijkers and Van Muiswinkel, 1977).

There is differential capacity in fish to respond to thymus-dependent (TD) and thymus-independent (TI) antigens (Tatner, 1986, 1996). This is because of differences in the time of maturation of cell populations involved in the humoral immune response. If common carp are exposed to a TD antigen before two months' of age, fish develop tolerance to this antigen (Van Loon *et al.*, 1981). A primary immune response to TD and TI antigens was observed in two month old common carp (Manning *et al.*, 1982). In rainbow trout, the ability to respond to TI antigen preceeds the ability to respond to TD antigen (Etlinger *et al.*, 1979). In rosy barb, specific humoral immune response to TD antigen was observed at the age of four months but the response did not reach adult level before nine months (Rijkers and Van Muiswinkel, 1977).

Very little is known about the effects of ageing on the immune response in fish. Different fish species have widely varying life spans from only a year in some tropical species to several years for salmonids. So, it is reasonable to expect that there will be

age related changes in their immune response. It is probable that as fish age there will be a decline in both non specific and specific immune response with the TD and TI components declining differentially (Tatner, 1996).

1.2.5.2. Hormones

Hormones play a major role in parr-smolt transformation in salmonids with direct effects on their immune system. During smoltification in coho salmon, *Oncorhynchus kisutch* a decrease in the number of lymphocytes and a lower specific humoral antibody response was observed (Maule *et al.*, 1987). These changes were probably due to the direct action of increased level of cortisol at that time (Tatner, 1996).

Sexual maturation and breeding can also have an effect on the immune response of fish not only by the direct or indirect hormonal effects but also in terms of partitioning of energy resources (Tatner, 1996). Development and maintenance of carotinoid based secondary sexual traits may have costs with regards to immunity as observed in Arctic char *Salvelinus alpinus* (Skarstein and Folstad, 1994).

1.2.5.3. Stress

The immune system, like other physiological systems is compromised by poor health. Stressful circumstances are known to depress specific aspects of immune responsiveness (Ellis, 1981). The stress response involves physiological and behavioural reactions, which may help the fish to adapt to a new situation but if the stress is severe or prolonged, it may exceed the capacity of fish to adjust resulting in a general breakdown of the immune system. Fish can be stressed as a result of poor water quality e.g. low

oxygen, high ammonia from excrement or suspended solids. The suppressive effects of stress on the immune response are mediated by hormones, principally the corticosteroids. High population density is another important factor, which besides having significant repercussion on water quality may also suppress the immune system. Many fish species which live in groups such as the European eel *Anguilla anguilla*, organise themselves into dominance hierarchies and subordinate members have persistently high levels of cortisol in blood. Poor growth rate was observed in such individuals and in some cases death might occur due to extreme stress (Peters and Hong, 1985). In common carp, only the dominant members in a population produced antibodies to an experimental trypanosome infection (Barrow 1955: cited by Ellis, 1988).

There is an increasing concern about stress management in intensive fish farming and culture practices such as handling, sorting, grading, transport are recognised as potential stress inducing agents of fish (Ellsaesser and Clem, 1986). If the animals' defence system is compromised by stress, it becomes increasingly susceptible to disease and even protection afforded by vaccination can be overwhelmed. In general, piscine response to stress shows many similarities to that of terrestrial vertebrates (Wendelaar Bonga, 1997). Oxygen uptake can be stimulated and behavioural changes may occur. A number of effects are primarily related to the aquatic environment such as an increasing permeability of the surface epithelia including the gills leading to hydromineral disturbances (Iger *et al.*, 1995).

Further to the direct effect of stress resulting from crowding some species of fish release pheromones when stressed and thus induce a stress response in others. This

effect results in a reduced immune response to infectious pancreatic necrosis virus (IPNV) in blue gourami *Trichogaster trichopterus* (Perlmutter *et al.*, 1973) and depression of heart and growth rate in common carp (Ellis, 1981). Stress hormones are also released by Mozambique tilapia, causing the release of pharmacologically active substances in other individuals which may result in their death (Henderson-Arzapalo and Stickney, 1980). There is growing evidence of interaction between the neuroendocrine system and immune system in fish, which may lead to immune suppression under stress conditions (Fevolden *et al*, 1994; Espelid *et al.*, 1996; Weyts *et al.*, 1997).

1.2.5.4. Temperature

Among the best studied and most significant factors affecting the specific immune response in fish, both cell mediated and humoral, in fish is temperature. Generally, the higher the temperature in the physiologically tolerated range, the faster the onset of response and the higher its magnitude. At low temperature, there is either a prolongation of the induction period with low response or a complete absence of response. The critical temperature for the development of an immune response in fish appears to be related to the natural environmental temperature experienced by a particular species. The best responses are obtained at the normal summer temperature of the species concerned (Ellis, 1982, 1988). Temperature which are low within the normal physiological range of a species of fish (less than 6°C for rainbow trout and 12°C for common carp can depress the immune response (Avtalion, 1981).

Only certain phases of the specific immune responses are temperature dependent. The first temperature dependent phase is thought to be the interaction between T and B

lymphocytes and this is disrupted either by blocking T helper function or by increasing T suppressor activity. The subsequent multiplication and differentiation of activated antibody producing B lymphocytes is temperature independent with a final temperature dependent phase involving the level of antibody production by plasma cells (Ellis, 1982). At low temperature anabolic activity of B lymphocytes is lowered (Azzolina, 1978) and the degree of saturation of fatty acid into T lymphocyte plasma membrane phospholipid is decreased, leading to a change in membrane fluidity and suppression of T lymphocyte activity (Abruzzeni *et al.*, 1982). In channel catfish, virgin T cells, rather than memory T cells, B cells or accessory cells are particularly susceptible to inhibitory influences of lower temperatures on specific immune responses (Bly and Clem, 1992).

In common carp, secondary immune response can be elicited at a lower temperature if the primary immunisation was done at high temperature. The temperature dependent phase of primary response occurred during the first few days after immunisation thereafter the antibody production was independent of temperature (Avtalion *et al.*, 1976). In the same species Rijkers *et al.* (1980a) reported successful primary antibody production between temperature of 8°C- 28°C but secondary response was absent in fish immunised below 18°C. Some degree of acclimatisation at low temperature has been reported in goldfish *Carassius auratus* but the magnitude is lower than fish maintained at optimum temperature (Azzolina, 1978).

1.2.5.5. Seasonal effects

Many poikilotherms possess endogenous rhythms and even when environmental conditions of photoperiod and temperature are kept constant, non-maturing animals still exhibit seasonally correlated variability of antibody response (Hussein *et al.*, 1979).

Under constant photoperiod and temperature, immature rainbow trout had lower antibody response to *Aeromonas salmonicida* when immunised prior to winter in comparison to immunisation prior to spring (Yamaguchi *et al.*, 1980). The mechanisms of these endogenous rhythms are not clearly understood.

1.2.6. Variation in disease resistance in fish populations

The fact that fish or a group of fish resists pathogens is an extremely complex phenomenon, which may have multiple reasons varying from one fish or one group of fish to another. Significant differences in survival among different closely related species, between populations and within population after specific diseases have been reported.

Relative susceptibilities of different fish species in aquaculture to major pathogens are well determined. Susceptibility of salmonids to *Ceratomyxa shasta* showed important variation in mortality. Mortality was almost total in rainbow trout, cutthroat trout *Oncorhynchus clarki clarki* and brook trout *Salvelinus fontinalis* whereas mortality was reduced in sockeye salmon *Oncorhynchus nerka* and almost non-existent in Atlantic salmon (Zinn *et al.*, 1977). Cultured in the same farm, brown trout *Salmo trutta*, brook trout and rainbow trout showed difference is susceptibility to furunculosis (Cipriano and Heartwell, 1986).

Variations in disease resistance between populations of farmed and natural populations have been found. Different populations of brook trout were reported to have different susceptibility to furunculosis and ulcer disease (Snieszko, 1959: cited by Chevassus and Dorson, 1990). Differences in susceptibility to furunculosis was observed among different hatchery strains of brown trout (Ehlinger, 1977) and rainbow

trout (Cipriano, 1983). Slight variation in mortality to vibriosis was observed in 14 wild populations of Norwegian Atlantic salmon whereas a higher mortality was observed in a Swedish population tested under the same conditions (Gjedrem and Aulstad, 1974). Hines *et al.* (1974) found two inbred lines of common carp to be highly susceptible to either epidermal epithelioma infection or swim bladder infection by *Aeromonas liquefaciens* and other two inbred lines were not infected. Considerable differences in resistance to infectious pancreatic necrosis (IPN) among strains of brook trout (Silim *et al.*, 1982), Japanese hatchery strains of rainbow trout (Okamoto *et al.*, 1987) and strains of channel catfish to channel catfish virus (CCV) have been found (Plumb *et al.*, 1975). A study by Zinn *et al.* (1977) found differences in susceptibility to parasite *Ceratomyxa* in hatchery strains of chinook salmon *Oncorhynchus tshawytscha.* Differential susceptibility to monogenean *Gyrodactylus salaris* in Altantic salmon stocks has been reported (Bakke *et al.*, 1990).

Significant variations are frequently found between groups of full sibs such as susceptibility of Atlantic salmon parr to vibriosis (Gjedrem and Aulstad, 1974), susceptibility of sockeye salmon *Oncorhynchus nerka* to infectious hematopeotic necrosis (IHN) (Amend and Nelson, 1977; McIntyre and Amend, 1978) and incidence of white spot *Ichthyoptherias multifilis* infection in southern platyfish *Xiphophorus maculatus* (Price and Bone, 1985). However, a genetic interpretation of this variation is difficult because in addition to additive genetic effects that can be selected for, there may be dominance and maternal effects. The existence of such maternal effects up to a certain age has been found in southern platyfish (Price and Bone, 1985).

1.2.7. Measurement of disease resistance in fish

In a breeding programme the economically important traits included in the breeding goal must be defined and the measurement of each trait must be done meaningfully. Disease resistance can possibly be measured by the following ways (Fjalestad *et al.*, 1993):

1.2.7.1. Survival rate

Survival rate, a desirable breeding goal in dealing with resistance, is a complex trait. It may be influenced by various pathogens. The level of survival may also depend on many irrelevant factors in disease resistance such as accidents and management problems. The effects of various pathogenic and non-pathogenic factors may vary between farms and between years in the same farm. Recording survival rate requires a marking system, which allows identification of dead fish along with individual diagnosis. For an individual fish, survival can only be recorded as dead or alive. For a group of individuals, such as full or half sibs, the mean survival rate in each group can be recorded. For a large number of groups, survival would tend to be normally distributed. When applying family selection as opposed to individual selection, the intensity of selection can thus become independent of the rate of survival and the genetic progress will be maximised when the overall rate of survival is 50% (Fjalestad *et al.*, 1993).

1.2.7.2. Immunological or physiological parameters

All factors that enable fish to resist pathogens in a specific environment are entities of the disease resistance complex. Immunological or physiological parameters from

healthy and diseased fish can be obtained from blood samples and compared as indirect measurements of disease resistance. Such parameters can be mechanical and chemical factors such as skin, mucus (Fletcher, 1978; Ingram, 1980; Cipriano and Heartwell, 1986), lysozyme (Fletcher and White, 1976; Roed *et al.*, 1989,1993a,b; Lund *et al.*, 1995b), interferon (de Kinkelin and Dorson, 1973) or proteolytic enzymes (Hjelmeland *et al.*, 1983), which constitute a first non-specific line of defence against pathogens in fish. Another important component is the complement system (haemolytic activity) which involves both specific and non-specific immune systems (Harrell *et al.*, 1976; Nonaka *et al.*, 1981; Sakai, 1981; Roed *et al.*, 1992; Slierendrecht *et al.*, 1993, 1996; Wiegertjes *et al.*, 1993b; Hollebecq *et al.*, 1995). An increasing number of studies have reported on the use of specific antibody levels and total antibody activity (total IgM) in fish to pathogenic organisms to detect correlation with disease resistance (Stromsheim *et al.*, 1994a,b; Eide *et al.*, 1994; Gjedrem *et al.*, 1991; Lund *et al.*, 1995a,b; Gjedrem and Gjoen, 1995). Among the physiological parameters cortisol has been widely used as indicator of disease resistance (Refstie, 1982; Fevolden *et al.*, 1991, 1992, 1994). All these parameters have shown genetic variation and their value in a breeding programme depends on their heritabilities and genetic correlation to survival (Fjalestad *et al.*, 1993).

1.2.7.3. Challenge test

Disease resistance can be recorded by exposing the fish to specific disease agents (Van Muiswinkel *et al.*, 1990; Gjedrem *et al.*, 1991; Ibarra *et al.*, 1991; Refstie *et al.*, 1993 cited by Van Muiswinkel, 1999; Stromsheim *et al.*, 1994a,b; Eide *et al.*, 1994; Gjedrem and Gjoen, 1995). Survival in experimental challenge and physiological and

immunological parameters are often used together as indicators of immune status and disease resistance (Cipriano and Heartwell, 1986; Hamilton *et al.*, 1986; Fevolden *et al*, 1991, 1992, 1994; Balfry *et al.*, 1994; Gjedrem *et al.*, 1991; Gjedrem and Gjoen, 1995; Lund *et al.*, 1995b; Wiegertjes *et al.*, 1995a,b; Slierendrecht *et al.*, 1993, 1996). To reduce costs, these challenge tests are often performed on small fish and over a short period of time. Challenge tests take place in closed facilities without the risk of infecting the breeding stock, commercial fish farms or wild populations. The environmental conditions under challenge tests differ from those in commercial farms and the efficiency of a challenge test may be reduced. The response to selection in the field will depend on the genetic correlation between survival after challenge test and general resistance in commercial farms.

1.2.8. Approach to improve disease resistance

1.2.8.1. Direct selection

High fecundity and external fertilisation are advantages in fish breeding. It is possible to produce groups of fish according to required experimental designs and each group of fish can be split into subgroups and tested for different characters under varying environmental conditions. Selection schemes should be designed to use either direct selection or indirect selection for correlated traits to disease resistance.

Significant differences in survival between closely related species, between populations and between full and half sib families in relation to specific diseases have been reported and already mentioned in Section 1.2.6. Species and populations showing resistance to disease are important candidates for direct selection. Some selection experiments to reduce mortality have shown responses, such as, selection for increased

resistance to furunculosis in brook trout and brown trout (Ehlinger, 1977; Capriano and Heartwell, 1986) and dropsy in carp (Kirpichnikov *et al.*, 1979).

1.2.8.2. Indirect selection

In a selection programme it may be desirable to include correlated traits with no economic value in order to increase the genetic gain in traits of importance (Gjedrem, 1967). Immunological and physiological parameters mentioned in Section 1.2.7.2. have shown genetic variation and are possible traits for indirect selection. Brown trout selected for high level of mucus precipitin against *A. salmonicida* were found to be more resistant to furunculosis than the unselected line (Cipriano and Heartwell, 1986). Atlantic salmon and rainbow trout lines were selected for high and low stress response as measured by blood cortisol level. Rainbow trout line selected for low cortisol response survived better following an experimental furunculosis challenge (Fevolden *et al.*, 1991). Increased mortality was found in the high cortisol response Atlantic salmon line (Fevolden *et al.*, 1992).

1.2.8.3. Hybridisation and crossbreeding

The use of hybridisation, which is frequently used in plants, is limited in fish because of viability of possible hybrids (Chevassus and Dorson, 1990). Ord *et al.*, (1976) reported that the hybrid between female rainbow trout and male coho salmon showed the paternal trait of resistance to viral haemorrhagic septicemia (VHS). The same hybrids were susceptible to IPN although coho salmon is resistant to IPN. The transmission of the resistance trait is not systematic in this hybrid (Dorson, 1987 cited by Chevassus and Dorson, 1990). Though hybrids between American catfish *Ictalurus furcatus* and

channel catfish are viable, they are susceptible to CCV like both parents (Smithermann *et al.*, 1983). Hybrids of common carp and goldfish inherit the susceptibility of gold fish to dropsy (Pojoga, 1972: cited by Chevassus and Dorson, 1990).

Breeding programmes frequently include crossbreeding between strains or lines to utilize heterosis. Several studies on the behaviour of disease resistance traits, observed between populations of same species, were done in crosses between populations. Crosses between different strains in carp have shown heterosis effects for survival (Hines *et al.*, 1974; Sovenyi *et al.*, 1988). Better survival in viral disease has been reported in American catfish crosses (Plumb *et al.*, 1975). Inter-strain crosses in salmonids have given variable results (Klupp, 1979; Ayles and Baker, 1983; Gjerde and Refstie, 1984).

Between population crosses often exhibit significant heterosis for growth and that factor may play a role in general resistance to disease. However, unlike improvement through additive genetic effects, it is very difficult to predict the results of crossbreeding. Thus there is no general answer as to whether or not crossbreeding should be done to increase the survival rate in fish farming. Several inter-species crosses yield progenies resistant to specific disease in some instances. However, due to low viability of these hybrids, they are less interesting for commercial farming (Chevassus and Dorson, 1990). Specialised lines for crossbreeding purposes can be developed if negative genetic correlations are found between disease resistance and other traits in the breeding goal. The heterotic results of such breeding strategies must be great enough to balance the extra cost of developing and maintaining such specialised lines (Fjalestad *et al.*, 1993).

1.2.8.4. Vaccination and use of antibiotics

Vaccine development ideally starts with the identification and characterisation of protective antigens derived from the causative agent of the disease. Implementation of vaccines depends not only on the demonstration of protection in the laboratory where the safety and efficacy of a vaccine is tested in the target species but also in the field performances under the farming conditions for which it is intended. The predominant method for assessing vaccine efficacy and potency is experimental infection using the fish species concerned (Van Muiswinkel *et al.*, 1999). For certain diseases, different routes of infection yield rather divergent results with regard to protection. Intraperitoneal (i.p) challenge is often inappropriate for evaluation of vaccine, which has been administered in the same way because of the crucial role of mucosal immunity. In such a case cohabitation challenge is more appropriate (Midtlyng, 1996).

Despite impressive technical progress, the number of commercially available vaccines remains limited and most of those are against bacterial diseases (Adams *et al.*, 1997). Although commercial vaccines have been available for many years, only recently have some has shown to be reproducible and highly protective against infectious disease such as furunculosis (Marsden and Secombes, 1997). While the efficacy has been improved, in the majority of cases the actual mechanisms of protection remain elusive (Giorgetti, 1991; Secombes and Olivier, 1997). The effectiveness of a vaccine should not be measured in terms of protection alone but also by its ability to elicit a specific immune response in fish to protective antigens (Hastings, 1988). Antibody titre has been measured following vaccination but often did not correlate with protection (McCarthy *et al.* 1983; Olivier *et al.*, 1985). However, some investigations have shown

antibody titres to protective antigens to be correlated with protection (Hirst and Ellis 1994; Ellis, 1997). One of the problems with viral vaccines is the relative high cost of traditional virus production in tissue culture systems. Current efforts to develop new viral vaccines have turned largely to recombinant DNA technology (Lorenzen and Olsen, 1997). Viral subunits containing antigenic determinants for the production of neutralising antibodies or activated T cells, but not harbouring infectious nucleic acid, have received wide acclaim as they will be safe to use. This technology could be the method of choice to produce large quantities of immunoprotective antigens in future (Van Muiswinkel *et al.*, 1999).

Antibiotics are known to affect the immune response in mammals but in fish, the effects are variable depending on the antibiotic, the aspect of use and the species of fish. The effect may be enhancement, suppression or none (Ellis, 1988). In common carp and in rainbow trout, oxytetracyclin suppressed specific humoral immune response to rabbit erythrocytes (Rijkers *et al.*, 1980b) and *Yersinia ruckeri* (Van Muiswinkel *et al.*, 1985) respectively but development of protection in rainbow trout against vibriosis was not supressed (Thorburn *et al.*, 1987). Oxytetracycline and oxolinic acid significantly supress antibody production as well as the level of circulating lymphocytes in rainbow trout when administered in association with immunisation. The phagocytic activity of the whole blood leucocytes was stimulated by oxolinic acid but slightly suppressed by oxytetracycline (Lunden *et al.*, 1998). Sulphonamide had no suppressive effect on rainbow trout against *Y. ruckeri* (Anderson *et al.*, 1984).

1.3. Tilapia as a model fish in research

McAndrew (2000) defined 'tilapia' as "a common name for large number of species within the cichlid tribe Tilapiini, particularly the species in the three genera *Tilapia*, *Sarotherodon* and *Oreochromis*, especially the larger species most commonly caught in wild fisheries or those used in aquaculture."

Records on tilapia culture showed that only eight to nine species of tilapia have significant potential in aquaculture (Schoenen, 1982; Pullin, 1983). Among them two species of the genus *Tilapia* (*T. zillii* and *T. rendalli*) and four species of the genus *Oreochromis* (Nile tilapia *O. niloticus*, Mozambique tilapia *O. mossambicus*, blue tilapia *O. aureus* and three spotted tilapia *O. andersonii*) are widely used in aquaculture. However, the Nile tilapia is by far the most important species in the group in freshwater tropical aquaculture (FAO, 2002).

The popularity of Nile tilapia culture derives from their physiological properties. Nile tilapia can tolerate a wide range of environmental conditions, such as high salinity, low oxygen and overcrowding, in addition to their obvious high growth rate on natural and cheap artificial feed. It is omnivorous and mainly feeds on phytoplankton, several species of zooplankton and macrophytes, and is also well adapted to supplementary feed. Their feeding regime varies considerably depending on their size, age, availability of feed and the presence or absence of competing species within their habitat. Fry feed on small organisms and detritus, and are thought to be cannibalistic (Philippart and Ruwet, 1982; Ajuzie and Nwokorie, 1994; Macintosh and Little, 1995).

Shorter generation times and the ability to breed in captive conditions are also added reasons behind its popularity. However, their habit of prolific breeding and cannibalism

in the culture ponds, has hindered aquaculture production of this species in many tropical countries. In the tropics, tilapia can become sexually mature and begin to reproduce at an age of five months, sometimes even earlier (Tave, 1993). This reproductive efficiency of tilapias results in subsequent unwanted reproduction leading to over-population, which in turn reduces the quality of the products as well as profits. To overcome this situation, the culture of single sex tilapia, preferably male because of their higher growth rate, has been adopted in many of the commercial production systems (Penman and McAndrew, 2000; Edwards *et al.*, 2000).

1.3.1. Infectious diseases of tilapia

Tilapias, in general, have an unusual relationship with pathogenic organisms. Few infectious agents are specific for tilapia. However under certain conditions some pathogens affect tilapia more severely than any other species of fish. Uncrowded tilapia under an optimum environment, especially with regard to temperature, remains relatively free of either bacteria or parasitic diseases. If tilapia become stressed due to low temperature, improper handling, exposure to poor water quality such as low oxygen or high ammonia, nitrite or carbon dioxide, adverse pH or overcrowding accompanied by high feeding rate, they become susceptible to a wide variety of infectious agents. Extensively or semi-intensively cultured tilapias in their natural warm water habitat usually do not contract serious diseases. Tilapia culture has become increasingly intensive and has expanded into temperate and colder climates where it is more difficult to maintain a proper environment by artificial means. Consequently infectious diseases have become increasingly more serious (Plumb, 1997).

Bacterial diseases are among the most serious infectious problems of cultured tilapia. No pathogenic bacteria are specific for tilapia but some affect them more severely than they do other cultured fish. The most common pathogenic bacteria and bacterial disease of tilapia are *Aeromonas hydrophila* and related species causing motile Aeromonas septicemia (MAS), *Vibrio anguillarum* and related species causing vibriosis, *Flavobacterium columnarae* causing columnaris disease, *Edwardseilla tarda* causing Edwardsiellosis, *Streptococcus* spp. and *Enterococcus* sp. causing Streptococcal disease (Roberts and Sommerville, 1982). Streptococcal disease caused by *Streptococcus iniae* is responsible for significant losses to the world aquaculture industry, especially the production of warm water species such as tilapia causing an estimated US$ 150 million in losses annually (Shoemaker and Klesius, 1997). Other bacteria pathogenic to tilapia include *Plesiomonas shegalloides* (Faisal and Popp, 1987), *Mycobacterium fortuitum* and *M. marinum* causing mycobacteriosis (fish tuberculosis) and rickettsia like organism (RLO) (Chen *et al.*, 1994). Fungal infection usually increases in tilapia as water temperature drops to near lethal level. *Saprolegnia* and *Branchiomyces* spp. are the most common fungi infecting tilapia (Roberts and Sommerville, 1982).

Tilapias are parasitised by external protozoa and monogenean trematodes but also may have internal parasites. Internal parasites generally cause little concern. External parasites are common on the body surface, fins and gills of cultured tilapia, specially those cultured in intensive systems or outside their normal habitat (Plumb, 1997). Among protozoan parasites, ciliates *Ichthyophthiriasis multifilis* (Subasinghe and Sommerville, 1992), flagellates *Cryptobia branchiophylis* (Natividad *et al.*, 1986) and *Ichthyobodo necatrix* (Plumb, 1997), sporozoans *Myxosoma tilapia* (Faisal and Shalaby,

1987); parasitic worms, monogenean trematode *Cichlidogyrus* sp. (Natividad, 1986), *Gyrodactylus*, *Dactylogyrus* (Plumb, 1997) and parasitic crustacea including anchor worm *Lernaea*, fish lice *Ergasilus* and *Argulus* cause considerable damage to cultured tilapia (reviewed by Plumb, 1997).

1.3.2. Immunological studies on tilapia

Most studies on tilapia disease focused on identification of tilapia pathogens and their effects and few immunological studies have involved tilapia. Sailendri and Muthukkaruppan (1975a,b) studied the lymphoid organs and immune responses in Mozambique tilapia. Ruangpan *et al.* (1986) studied the protective efficacy of formalin killed *A. hydrophila* vaccine in Nile tilapia. The immune response of the same species after hyperosmotic infiltration vaccination against *E. tarda* was studied by Lio-Po and Wakabayashi (1986). Mochida *et al.* (1994) studied the physical and biochemical properties of Nile tilapia IgM. Sarder (1998) studied the genetic aspects of specific and non-specific immune responses in Nile tilapia clonal lines. Studies on experimental *Streptococcus iniae* infection in Nile tilapia were done by Evans *et al.* (2000) and Shoemaker *et al.* (2000). Monoclonal antibodies (MAbs) against Nile tilapia immunoglobulin have been produced and characterised (Al-Harbi *et al.*, 2000; Chowdhury *et al.*, pers. comm.). These MAbs could be vital tools to monitor humoral antibody response in Nile tilapia following natural infections or vaccination and to evaluate antigen recognition by immunoblotting (Al-Harbi *et al.*, 2000).

1.4. Importance of inbred strains in immunological assays

Inbred laboratory fish lines, selected for high or low immune responsiveness and in which these phenotypes have clearly been defined, can offer appropriate models for investigating the genetic factors of resistance to infections, similar to the role played by comparable mouse lines (Mouton *et al.*, 1988). Such inbred lines can have a clear experimental value in unravelling environmental and genetic effects and their stable genetic constitution can be invaluable for a thorough immunological analysis of disease resistance. They will allow comparison of data between subsequent generations (Wiegertjes *et al.*, 1994).

Current methods to control infectious disease in fish consists of hygiene, vaccination, medication and eradication of infected population. Improving infectious disease resistance by genetic means is an attractive alternative approach because of its prospects for prolonged sustainable production. It has been thoroughly established that both cold and warm blooded vertebrates' ability to resist infection by a wide range of pathogens is influenced by genetic factors (Chevassus and Dorson, 1990; Skamene and Pietrangeli, 1991). Consequently, outbred host population will inevitably show variability in their natural immunity to infection (Wiegertjes *et al.*, 1996a).

There are a number of approaches to study of genetic influences on disease resistance in animals (Festing and Blackwell, 1988). One approach is selective breeding for changes in immune responsiveness. This approach is used especially when disease resistance is expected to be under polygenic control. Selective breeding focuses on families homozygous only for those genes determining the immune response under selection. Variability within and between lines is required to unravel polygenic control.

This approach can be used for animal species with short generation intervals only because as many as fifteen generations may be needed to attain homozygosity i.e. fixing or clustering of favourable/unfavourable genes in a selection line. Inbred fish lines can be a valuable tool for the approach because as fish are lower vertebrates, the quality of the immune response is also controlled by polygenic factors (Cossarini-Dunier, 1986).

Another strategy aimed at a full genetic analysis of host resistance, is the examination of a large number of inbred animal lines for a pattern of resistance which can be accounted for by a single genetic locus. On establishment of such a pattern, classical crosses including F1 hybrids, F2 hybrids and back crosses to both parental lines can be used to demonstrate Mendelian inheritance, followed by linkage studies with known genetic markers to map the Mendelian locus on the chromosome (Festing and Blackwell, 1988).

Many high yielding and disease resistant strains have been developed for agriculture in the last few decades by selection and genetic manipulation. Chromosome set manipulations have been widely used in research related to aquaculture, however, there are relatively few direct applications for genetic improvement of aquaculture stocks (Sarder, 1998).

By manipulating the chromosome sets, it has been possible to produce haploids, polyploids (triploids and tetraploids), gynogenetic (meiotic and mitotic) and androgenetic diploids in many fish spercies. Polyploids, particularly triploid fish has great potential in applied aquaculture whereas gynogenetic and androgentic fish have applications mostly in applied research. However, development of disease resistance strain of fish is a promising objective of genetic manipulation techniques. As mentioned

earlier, fish have a distinct immune system which is controlled by a group of polymorphic genes called the major histocompatibility complex (MHC) (Rijkers, 1982). In addition fish might also possess other genes which also control the immune response. Thus, the genes involved in immune response can be manipulated by producing homozygous inbred and outbred clonal lines using either gynogenesis or androgenesis. Since inbred lines are homozygous for every gene locus for a particular allelic set of genes, they may differ from each other and show variable disease resistance. By genetic manipulation disease resistant traits could also be manipulated from one fish species to another by gynogenesis using irradiated sperm. The offspring produced by this way would contain residual parental inheritance which may show resistance to certain diseases and has been reported in rainbow trout (Thorgaard *et al.*, 1985; Disney *et al.*, 1987).

1.5. Fish clones

Clones are genetically identical individuals. Clonal reproductive propagation is a common process in many lower vertebrates. Approximately 70 clonally reproducing, vertebrate biotypes of fishes, amphibians and reptiles have been identified and the majority (64%) are polyploids mostly 3n or 4n (Vrijenhoek *et al.*, 1989). Genetic studies have revealed that clonally reproducing, all-female 'species' of vertebrates are the products of hybridisation between congeneric sexual species. A new unisexual (all-female) lineage can be established if the genetic differences between the hybridising entities are sufficient to disrupt recombinant processes during meiosis, without severely limiting viability, fecundity, and other characteristics affecting fitness of the hybrids. With such a narrow window of opportunity, the origins and establishment of new unisexual vertebrates have been ecologically, geographically, and phylogenetically constrained (Vrijenhoek *et al.*, 1989, Vrijenhoek, 1994).

Sterility, often associated with hybridisation, provides strong selection pressure for any oogenetic mechanism that rescues egg production and retains or restores diploidy (Schultz, 1969). If such hybrids are viable and fertile, an 'oogenetic rescue mechanism' will rapidly be fixed if demographic advantages also arise with all-female reproduction (Vrijenhoek, 1994). Thus it is not surprising that several modes of clonal reproduction are found among unisexual vertebrates, such as parthenogenesis, gynogenesis and hybridogenesis, and that the cytological mechanisms underlying these modes may be unrelated (reviewed by Cuellar, 1977; Quattro *et al.*, 1992; Vrijenhoek, 1994; Carmona *et al.*, 1997).

Among the vertebrates, true parthenogenesis occurs in unisexual lizards. Sperm-

dependant, non-recombinant modes of reproduction, gynogenesis and hybridogenesis occur in fish and amphibia (reviewed by Vrijenhoek, 1994).

The first unisexual clonal vertebrate discovered was the Amazon molly *Poecilia formosa* (Hubbs and Hubbs, 1932: cited by Vrijenhoek, 1994). Since the discovery of this species in 1932, the study of these fish and their sexual relatives has contributed to our understanding about: (i) the origins and evolution of asexuality; (ii) the ecology of hybrids; (ii) genotypic and environmental effects on ecologically relevant traits; (iv) the maintenance of sex in higher organisms; and (v) the role of genetic variation in the survival of small endangered populations. The results of these studies provided information about the significance of genetic diversity for survival in spatially and temporally heterogeneous environments. Moreover, these fish provided genetically uniform systems for many environmental and biomedical studies (Vrijenhoek, 1994).

Survival of clonal fish is usually very low. This might be due to expression of recessive deleterious genes in the homozygous progeny (Naruse *et al.*, 1985). Another possibility is damage caused by physical shocks to egg or sperm chromosomes, because in most fish species metaphase chromosomes at first cleavage are very fragile (Komen *et al.*, 1991). In addition the fraction of eggs at metaphase at the time of application of physical shock determines the number of eggs that can respond positively to the shock (Komen *et al.*, 1991). These manipulations also result in considerable inbreeding depression, in clonal fish, 50% in meiotic and 100% in mitotic fish and many fish are lost during weaning and maturation. As a result many mature clonal fish show severe defects in their gonads and only a few from a batch can be reproduced gynogenetically or androgenetically (Komen *et al.*, 1991).

1.5.1. Artificial induction and propagation of fully inbred (homozygous) and outbred (heterozygous) clonal lines in fish

Artificial induction of clonal lines in fish can be obtained using induced mitotic gynogenesis or androgenesis in the first generation and meiotic gynogenesis or a second round of androgenesis in the second generation. Clonal lines can also be established from naturally occuring all-female species by one round of meiotic gynogenesis. Once established these clonal lines can be propagated by sibmating using sex-reversed individuals. A brief description of the production and propagation of clonal lines using chromosome set manipulation techniques is given in the following sections.

Gynogenesis is a form of all maternal origin, while androgenesis is the form of all paternal origin. The technique of induced gynogenesis involves the fertilisation of eggs with genetically inactivated sperm (mostly by ultraviolet irradiation, UV) and subsequent diploidisation by the suppression of the second meiotic division (meiotic gynogenesis) or suppression of first mitotic division by physical shocks (either heat, cold or pressure) or chemical treatments. In contrast, the technique of induced androgenesis involves the fertilisation of genetically inactivated eggs (mostly by UV or ^{60}Co) and subsequent diploidisation by suppressing the first cleavage or by fertilising the genetically inactivated eggs with sperms from tetraploid males (Thorgaard *et al.*, 1990).

1.5.2. Production of fully inbred clonal lines by induced gynogenesis

Fully inbred clonal lines can be produced in two generations by induced gynogenesis. A schematic diagram is presented in Fig. 1.3. In the first generation, isogenic lines of fish can be produced by using mitotic gynogenesis. Fully inbred clonal lines can be established in the second generation using this isogeneic lines by meiotic gynogenesis

(Fig. 1.3). Due to persistent heterozygosity in meiotic gynogenetics it is not possible to produce completely homozygous individuals unless some form of sib mating is used in conjunction with this.

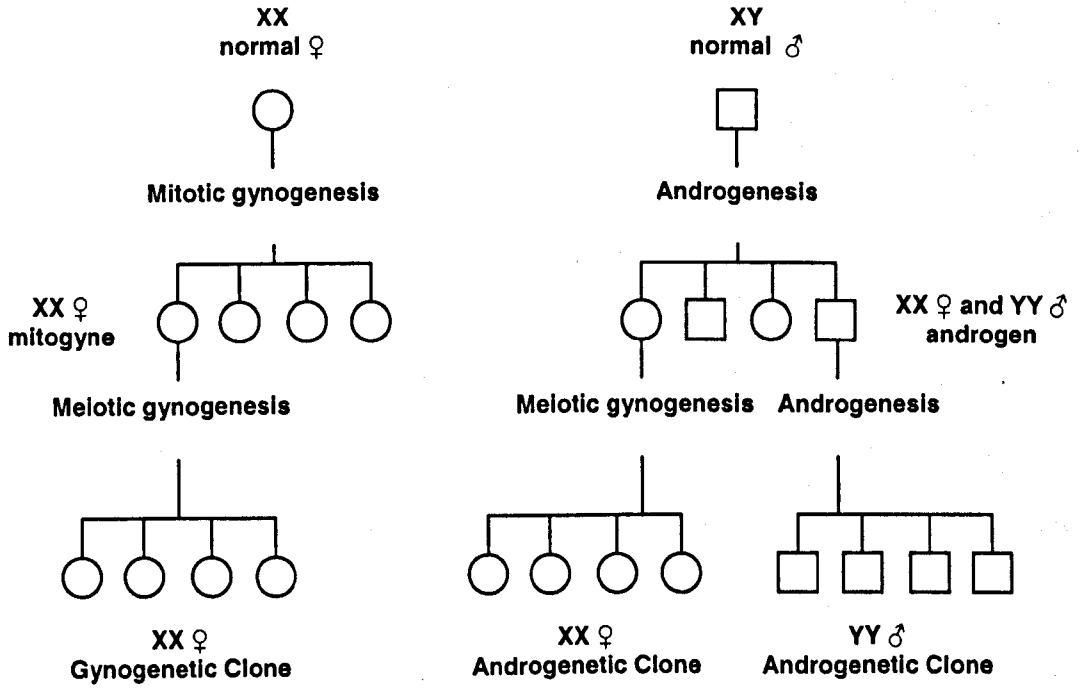


Fig. 1.3. Schematic representation of production of fully inbred (homozygous) clonal lines by gynogenesis and androgenesis.

In species with an XX/XY sex-determining system, gynogenesis usually produces all-female clonal lines. However, feminisation of XY males into neofemales and their subsequent use in mitotic gynogenesis will result in XX and YY isogenic fish in the first generation (Ezaz, 2002). In the second generation, meiotic gynogenesis with XX females and androgenesis with YY females would result in XX and YY clonal lines from same founder female. These techniques of combined sex reversal, gynogenesis and androgenesis might be used as an alternative method to produce XX and YY clonal lines by androgenesis.

Survival rates are usually very low among mitotic gynogenetics (Pandian and

Koteeswaran, 1998; Sardar *et al.*, 1999; Arai, 2001; Ezaz, 2002). Another problem of surviving mitotic gynogenetics is drastically reduced fertility with small numbers of mature fish, low fecundity, very poor egg quality and urinogenital deformities such as blocked urinogenital opening (Arai, 2001; Ezaz, 2002). Another potential constraint for the development of clonal lines is contamination by spontaneously occurring meiotic gynogenetics, which show better survival than induced mitotic gynogenetics which would result in a clonal progeny (Arai, 2001).

Until now, fully inbred gynogenetic clonal lines have been established in several commercially important and laboratory fish species including zebrafish *Danio rerio* (Streisinger *et al.*, 1981), medaka *Oryzias latipes* (Naruse *et al.*, 1985), common carp (Komen *et al.*, 1991), ayu *Plecoglossus altivelis* (Han *et al.*, 1991), hirame *Paralichthys olivaceus* (Hara *et al.*, 1993; Tabata, 1997; Yamamoto, 1999), amago salmon *Oncorhynchus rhodurus* (Kobayashi *et al.*, 1994), rainbow trout (Young *et al.*, 1996), Nile tilapia (Müller-Belecke and Hörstgen-Schwark, 1995; Sarder *et al.*, 1999) and red seabream *Pagrus major* (Kato, 1999). The advantage of using the technique of gynogenesis over androgenesis is due to the ease in handling of spermatozoa rather than eggs as well as a lack of large-scale facilities to inactivate large numbers of eggs at the same time.

Clonal lines can also be induced by gynogenetic development of spontaneously occurring unreduced eggs, which have been reported in some hybrids and unisexual fish (Vrijenhoek, 1994). Unreduced diploid eggs have been reported in hybrids between two different medaka species, *O. latipes* and *O. curvinotus* (Sakaizumi *et al.*, 1993) and also in hybrids between normal diploid and natural tetraploid cyprinid loach *Misgurnus*

anguillicaudatus (Matsubara *et al.*, 1995; Zhang and Arai, 1996). Arai and Mukaino (1997) observed that most gynogenetic triploid progeny in loach, which were induced by fertilisation of unreduced triploid eggs with UV-irradiated heterospecific spermatozoa, gave DNA fingerprints identical to the somatic cells of the triploid mother and thus were considered to be clones.

1.5.3. Production of fully inbred clonal lines by induced androgenesis

Like induced gynogenesis, induced androgenesis can also be used to produce homozygous clonal lines in fish in two generations. In some species male generation time is shorter than the female which suggests androgenesis could be quicker than gynogenesis to produce clonal lines in fish. A schematic diagram of the production of inbred clonal lines by induced androgenesis is presented in Fig. 1.3.

Although various sex-determining systems operate in different species of fish, the majority of the commercially important fish species show either XX/XY or WZ/ZZ sex determining systems. For example, starting with an XY male, XX and YY fish can be produced in the first generation by induced androgenesis. To generate homozygous clonal lines, meiotic gynogenesis with XX androgenetic females and another round of androgenesis with YY androgenetic males will produce XX all-female and YY all-male homozygous clonal lines (Fig. 1.3).

Unlike gynogenetic clonal line, fully inbred androgentic clonal fish have been reported only in few fish species including rainbow trout (Young *et al.*, 1996); common carp (Bongers *et al.*, 1995) and in amago salmon (Nagoya *et al.*, 1996).

1.5.4. Propagation of clonal lines

Most studies involving the production of mitotic gynogenetics or androgenetics reported very poor survival rate. However, because of the importance of clonal lines in various research areas, propagation of clonal lines by combining the technique of sex-reversal and subsequent sib-mating have been reported in several fish species. The process involved, either masculinisation (in case of gynogenetic clonal lines) or feminisation (in case of propagating androgenetic clonal lines) of a proportion of first generation clonal females or males by oral administration of steroid hormones. These sex-reversed neofemales or neomales can then be crossed to their sibs or their founder male or female for the rapid propagation of clonal lines. This technique of propagation of inbred clonal lines has been described in Nile tilapia (Sarder *et al.*, 1999).

1.5.5. Production of outbred clonal lines

Outbred or heterozygous clonal lines can easily be produced by crossing two inbred clonal lines. Hormonally sex-reversed neomales or neofemales from one inbred clonal line are crossed to clonal females or males from a different line to produce outbred clonal lines. Outbred clonal fish are also of potentially great importance in aquaculture to improve the stock by producing heterozygous stock with desirable, uniform, attributes, such as better growth performance and disease resistance. Outbred clonal lines have been produced in Nile tilapia and their performance has been tested in immunological assays (Sarder *et al.*, 2001)

1.5.6. Application of clonal lines in aquaculture

Clones are completely homozygous for every gene locus therefore they have potential for fixing superior genes (e.g. disease resistance). Thus a pure gene pool can be established in a clonal line which might be similar to the starting population. Survivivg clonal lines are supposed to be free from recessive lethal and major deleterious alleles. Thus they can be used in selective breeding programmes for improving fish stocks (Han *et al.*, 1991). However, clonal fish are very difficult to reproduce in most cases, which hinder their large-scale productions and applications.

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 expression can be examined in them. They are the identical offspring of a homozygous mother or father, so sex ratios of clones can be used to reveal the sex determining factors for a given species. Clones are supposed to respond to any kind of adverse environmental changes, therefore they can be used as test animals in toxicological bioassays and in vaccine development.

Although both inbred and outberd clonal lines have been established in a number of commercially important and laboratory fish species by chromosome set manipulations, most of these studies described the production, propagation and verification of clonal lines. Only a few reports are available where clonal fish have been used in analysis of various traits or bioassays.

Among these studies, most reported the growth and reproductive performances of clonal fish compared to that of the normal wild type populations such as in common carp

(Komen *et al.*, 1993; Bongers *et al.*, 1995) and Nile tilapia (Müller-Belecke and Hörstgen-Schwark, 2000). Del Valle and Taniguchi (1995) described the genetic variation of some physiological traits by using genetic variation index (GVI) as an indicator of heritability in clonal ayu under stressed and non-stressed conditions.

Only one report is available where inbred and outbred clonal lines have been used in immunological assays. Sarder *et al.* (2001) used both inbred and outbred clonal lines of Nile tilapia in analysis of non-specific immune responses. The authors examined the non-specific immune responses by analysing an array of immunological parameters such as lysozyme activity, number of macrophages and an artificial challenge with the pathogen A. *hydrophila.* They found significant differences in lysozyme activities among the inbred clonal lines, whereas outbred clonal line showed intermediate level of lysozyme activities to that of their parents. Similar results were found in the number of macrophages among the inbred and outbred clonal lines. In the case of artificial challenge with *A. hydrophila*, significantly different susceptibility to infection was observed among the inbred clonal lines with intermediate resistance showed by the outbred clonal lines. The outbred clonal lines also showed significantly higher resistance to *A. hydrophila* than the least resistant parental clonal line. From these results, the authors suggested that there might be a genetic difference in the resistance to *A. hydrophila* between the clonal lines and, therefore, the disease resistance of an outbred population might be improved by crossbreeding. In their studies they also studied the presence of MHC (major histocompatibility complex) type II genes by both PCR and scale grafting techniques. The allelic variation for these was detected between the clonal lines by PCR and differences between the clonal lines were also demonstrated

proved as graft rejection by reciprocal recipient fish.

1.6. Objectives of the study

The primary goal of the experiments described in this thesis was to compare the immune response and disease resistance of Nile tilapia clonal lines. Clones are completely homozygous for every gene locus and have potential for fixing superior genes. Clonal animals are preferred for immunological studies to control variation caused by differences in the genetic background of the experimental animals. In brief, the research focused on the following objectives:

1. Production, propagation and verification of Nile tilapia clonal lines.
2. Comparison of specific immune response and disease resistance of the clonal lines by using a number of different antigens and a pathogen.
3. Comparison of polyclonal activation of B and T lymphocytes in the different lymphoid organs of the clonal lines.

CHAPTER 2

PRODUCTION AND PROPAGATION OF HOMOZYGOUS AND HETEROZYGOUS CLONAL LINES IN NILE TILAPIA *Oreochromis niloticus*

2.1. Introduction

Homozygous lines of any laboratory animals have potential use in many fields of biological research, such as immunology and developmental biology. To produce such animals by conventional breeding methods such as by brother-sister mating require over 20 generations, which is both tedious and expensive (Naruse *et al.*, 1985). The experimental production of highly inbred lines of farm animals has been attempted without much success (Müller-Belecke and Hörstgen-Schwark, 2000). Clonal animals occur in natural populations of multicellular animals at all levels of organismic evolution, but within vertebrates they are confined to teleost fish, amphibians and reptiles (Schartl *et al.*, 1991). The external fertilisation and development in teleost fish and amphibians allow genetic manipulation using chromosome set manipulation during the embryonic development to produce homozygous or clonal populations within two generations.

Clonal fish induced by chromosome set manipulations are homozygous at all loci throughout the genome and are genetically identical. The chromosome set manipulation process involved in the production of such clonal lines are gynogenesis and androgenesis. As described in the Chapter 1, mitotic gynogenesis or androgenesis in the first generation and meiotic gynogenesis or androgenesis in the second generation produce fully inbred clonal lines of fish.

Until now, fully inbred clonal lines have been established in eight fish species including zebra fish (Streisinger *et al.*, 1981), medaka (Naruse *et al.*, 1985), common carp (Komen *et al.*, 1991), ayu (Han *et al.*, 1991), hirame (Hara *et al.*, 1993), amago

salmon (Kobayashi *et al.*, 1994), rainbow trout (Young *et al.*, 1996) and Nile tilapia (Müller-Belecke and Hörstgen-Schwark, 1995).

The Nile tilapia is one of the important aquaculture species and an increasingly popular laboratory animal model. Polyploid, gynogenetic (both meiotic and mitotic) and androgenetic Nile tilapia have been extensively used to study various fields of research including linkage mapping (Kocher *et al.*, 1998), sex determination (Mair *et al*, 1991a,b; Penman *et al.*, 1987a,b, Penman, 1989; Sarder *et al.*, 1999; Ezaz, 2002), growth and gonadal development (McAndrew and Majumder, 1989). Fully inbred (homozygous) and heterozygous (outbred) clones have been produced in this species by gynogenesis as well as by androgenesis and have been used in various studies including immunology, sex determination, reproductive and growth analysis (Sarder *et al.*, 1999, 2001; Jenneckens *et al.*, 1999; Müller-Belecke and Hörstgen-Schwark, 2000).

2.1.1. Gynogenesis

Gynogenesis is a special type of sexual reproduction in which insemination is obligatory but the sperm nucleus does not take part in embryological development, and the ovum nucleus, with an unreduced number of chromosomes, becomes the zygote nucleus. Natural gynogenesis is not uncommon in fishes and is found in certain unisexual populations. Some populations of crucian carp *Carassius auratus* and several species of small viviparous fishes of the family Poeciliidae (*Poecilia* and *Poecilopsis*) reproduce naturally by gynogenesis (Golovinskaya, 1968; Cherfas, 1981; Thorgaard, 1983).

2.1.1.1. Induced gynogenesis

The second meiotic division in fish eggs occurs after fertilisation, just before the fusion of the male and female pronuclei to form the zygote. During the normal course of cell division after fertilisation, either the second meiotic division or first mitosis can be manipulated or prevented in fish by applying suitable physical shocks (such as temperature or pressure) or treatment with some chemicals. Apart from physical shocks, several chemicals have been used to block polar body extrusion or mitotic division in fertilised eggs (Refstie *et al.*, 1977; Smith and Lemonie, 1979). Anaesthetics such as nitrous oxide and Freon have been used to block the first meiotic division (Johnstone *et al.*, 1989). In comparison to the success and ease of application of other methods, chemicals are not so suitable for mass production (Thorgaard, 1983).

The first successful example of artificial inactivation of sperm chromosomes without impairment of the sperm's fertilising capacity was described by Hertwig in 1911 in frogs (reviewed by Cherfas, 1981; Thorgaard, 1986; Ihssen *et al.*, 1990; Purdom, 1993; Hussain, 1996). He observed massive, early embryonic mortality at low doses of irradiation and massive but delayed mortalities at higher doses. The embryos resulting from the latter were all abnormal. This paradoxical phenomenon is known as the 'Hertwig effect'. The probable explanation of the 'Hertwig effect' lies in the partial inactivation of the sperm chromatin at low doses with the result that the embryos develop with an aneuploid chromosomal condition, which is detrimental to survival very early in development. At higher doses, however, the sperm chromatin is completely inactivated or destroyed, and the maternal haploid chromosomes participate in further development. This gynogenetic haploid condition seems to be far less deleterious to

development than aneuploidy (Ijiri and Egami, 1980). Opermann (1913: cited by Ihssen *et al.*, 1990) became the first author to test the 'Hertwig effect' in fish. He used ionizing rays of radium and thorium on the sperm of brown trout and observed a typical 'Hertwig effect'.

Sperm treated with radiation or chemicals usually show a lower viability and fertilising capacity than those treated with UV radiation (Chourrout, 1987). Application of appropriate UV to sperm before fertilisation is routinely used to destroy the paternal genome.

Haploid gynogenetic individuals are produced if such genetically inactivated sperm are used to fertilise eggs. Such haploid gynogenetic individuals normally survive until hatching but very few survive up to first feeding, with only one report of haploid gynogenetic Mozambique tilapia surviving up to the age of maturation of normal diploid control individuals (Varadaraj, 1993).

The combination of UV irradiation of sperm with an 'early' shock to prevent second polar body extrusion results in a form of diploid gynogenesis which is generally referred to as "meiotic" gynogenesis. However, the fertilisation of eggs with UV treated sperm and "late" shock treatment which restores diploidy by endomitosis also produces diploid gynogenetic individuals, which is referred to as "mitotic" gynogenesis.

Individuals originating from mitotic gynogenesis are completely homozygous because of the duplication of a single (haploid) chromosome set. However, within a single batch of eggs, individuals are not identical to each other because of the heterozygosity in the dam and the crossing over which occurred during the first meiotic division. In contrast, meiotic gynogenetics from an outbred mother are each partially

heterozygous, again depending on the level of heterozygosity in the mother and the degree of recombination occurring during meiosis due to crossing over (Nace *et al.*, 1970, Hussain *et al.*, 1994a; reviewed by Penman and McAndrew, 2000; Arai, 2001).

Meiotic gynogenesis has been achieved in many fish species, including grass carp *Ctenopharyngodon idella* (Stanley and Sneed, 1974; Stanley, 1976), plaice *Pleuronectes platessa* (Purdom *et al.*, 1976; Thompson *et al.*, 1981), common carp (Nagy *et al.*, 1978; Nagy and Csanyi, 1982; Hollebecq *et al.*, 1986; Komen *et al.*, 1988), zebrafish (Streisinger *et al.*, 1981), rainbow trout (Chourrout and Quillet, 1982; Thorgaard *et al.*, 1983; Chourrout, 1984; Lou and Purdom, 1984; Thompson and Scott, 1984; Kaastrup and Horlyck, 1987), Indian major carp *Labeo rohita* (John *et al.*, 1984), cyprinid loach (Suzuki *et al.*, 1985), African catfish *Clarias gariepinus* (Volckaert *et al.*, 1994, 1997) and silver barb *Barbodes gonionotus* (Pongthana *et al.*, 1995).

The earliest attempt to induce mitotic diploidisation by interfering with first cleavage of eggs in plaice was not successful (Purdom, 1969). The first successful diploidisation was reported in zebrafish by Streisinger *et al.* (1981). Afterwards this technique became more familiar to others and attempts were made to produce mitotic gynogenetics using different shock treatments such as heat shock (Purdom *et al.*, 1985) cold shock (Krasznai and Marian, 1987) and pressure shock (Chourrout 1984; Onozato, 1984; Naruse *et al.*, 1985). Successful mitotic gynogenesis has also been reported in a number of other species, including common carp (Nagy, 1987; Komen *et al.*, 1991), medaka (Ijiri, 1987), ayu (Taniguchi *et al.*, 1988), Indian major carp (Hussain *et al.*, 1994b) and African catfish (Galbusera *et al.*, 2000).

2.1.1.2. Gynogenesis in tilapia

Recent studies have attempted to induce gynogenesis in tilapia with the objective of elucidating sex-determining mechanism (Avtalion and Don, 1990; Pandian and Varadaraj, 1990; Mair *et al.*, 1991a,b). Induced gynogenesis has proven to be very useful in this respect. For instance, gynogenesis was used to confirm female homogamety in Nile tilapia and Mozambique tilapia, to detect gene-centromere recombination for the sex determining loci in blue tilapia and Nile tilapia (Penman *et al.*, 1987a; Avtalion and Don, 1990; Mair *et al.*, 1991a,b; Ezaz, 2002) and in the development of genetic linkage maps using haploid gynogenesis (Kocher *et al.*, 1998). Induced mitotic gynogenesis was also used to produce XX and YY isogenic lines in Nile tilapia from XY neofemales and to isolate sex-linked AFLP markers by bulked segregant analysis (Ezaz, 2002). This technique was also used to develop completely homozygous clones (Müller-Belecke and Hörstgen-Schwark, 1995; Hussain *et al.*, 1998; Jenenckens *et al.*, 1999; Sarder *et al.*, 1999) and heterozygous outbred clones in *O. niloticus* (Hussain *et al.*, 1998; Sarder *et al.*, 1999). Most of these studies concentrated exclusively on the commercially important *Oreochromis* species Nile tilapia, *blue tilapia* and Mozambique tilapia. There are no known reports of chromosome set manipulation studies in other genera of tilapia (Mair, 1993). Among the above mentioned three species most studies have been done in Nile tilapia.

Both meiotic (Chourrout and Itskovich, 1983; Penman *et al.*, 1987b; Mair *et al.*, 1987, 1991a,b; Varadaraj 1990a,b; Hussain *et al.* 1993; Sarder *et al.*, 1999; Müller-Belecke and Hörstgen-Schwark, 2000) and mitotic (Mair *et al.*, 1987; Hussain *et al.*, 1993; Myers *et al.*, 1995; Müller-Belecke and Hörstgen-Schwark, 1995; Sarder *et al.*,

1999) gynogenetic individuals have been produced in the above mentioned three *Oreochromis* species using different shock treatments (cold, heat and pressure). However, most researchers have used UV irradiation to inactivate the sperm genome. Varadaraj (1990a) and Peruzzi *et al.* (1993) also reported the production of gynogenetics in Mozambique tilapia and Nile tilapia by heterologous fertilisation using common carp, crucian carp and koi carp sperm.

Haploid gynogenetic individuals have been produced in all the three species of *Oreochromis*, and Don and Avtalion (1988) and Penman (1989) have observed a 'Hertwig effect'. Haploids are usually non-viable (with one exception reported by Varadaraj, 1993) in all three species, usually dying within 48 h post-hatching, and exhibiting a typical haploid syndrome such as shortening and deformation of the body, excessive hydration of the pericardium and impairment of pigmentation. Spontaneous diploidization of the maternal genome has been reported in Nile tilapia only in one occasion (Ezaz, 2002).

2.1.1.3. Verification of gynogenetic and clonal diploids

In many laboratories techniques of chromosome set manipulations are fairly routine and are used to produce homozygous fish. However, analysis of inheritance in genetic manipulation studies, such as gynogenesis, androgenesis and clonal fish, is important to evaluate the success of the procedure to avoid contamination in clonal lines. Phenotypic markers can be used if the trait is based on a recessive allele (Galbusera *et al.*, 2000) and such characteristics have been used to assess inheritance in gynogenetic fish such as carp (Nagy *et al.*, 1978) and tilapia (Don and Avtalion, 1988; Varadaraj, 1990a) and

androgenetic tilapia (Myers *et al.*, 1995). However, such morphological markers are rare, and, even if available, often require confirmation using biochemical or molecular markers to allow the unambiguous identification of inheritance.

Biochemical markers such as allozyme loci have been applied in several fish species to monitor genetic manipulation. Such species include chinook salmon (Levanduski *et al.*, 1990), ayu (Han *et al.*, 1991), carp (Yousefian *et al.*, 1996) and Nile tilapia (Hussain *et al.*, 1993; Müller-Belecke and Hörstgen-Schwark, 1995; Sarder *et al.*, 1999; Karayücel, 1999). Allozyme analysis has also revealed high levels of heterozygosity at a number of loci in meiotic gynogenetics (Hussain *et al.*, 1993; Sarder *et al.*, 1999). This technique can thus be an efficient tool in discriminating meiotic gynogenetic individuals from mitotic gynogenetic individuals (Hussain *et al.*, 1993; Sarder *et al.*, 1999). However, low levels of genetic variability are often observed using this technique, which limits its application (Ferguson and Danzmann, 1998).

The recent advances in DNA technology have provided many genetic markers and often result in the detection of high levels of genetic variation. Multilocus DNA fingerprinting using variable number of tandem repeats (VNTRs) (Tautz and Renz, 1984; Jeffreys *et al.*, 1985a,b) has been used in inheritance analysis studies of genetically manipulated populations of Nile tilapia (Carter *et al.*, 1991; Sarder *et al.*, 1999; Karayücel, 1999; Jenneckens *et al.*, 1999) and African catfish (Volckaert *et al.*, 1994).

2.1.1.4. Application of gynogenesis

The major rationale for the interest in gynogenesis has been the potential for

producing inbred lines for breeding programmes and research purposes (Golovinskaya, 1968; Purdom, 1969, 1993). Meiotic gynogenetics are partially heterozygous due to the occurrence of recombination between chromatids during the first meiotic division of the eggs (Purdom, 1969; Nace *et al.*, 1970; Hussain *et al.*, 1994a). Earlier studies indicated little crossing-over between genes and the centromere in fish chromosomes, resulting in low levels of heterozygosity (Purdom, 1969). Subsequent studies showed high levels of heterozygosity at some loci in meiotic gynogenetic diploids, was reported in common carp (Golovinskaya and Romashov, 1966), rainbow trout (Thorgaard *et al.*, 1983), brown trout (Guyomard, 1986) and Nile tilapia (Hussain *et al.*, 1994a). However, meiotic gynogenesis are very useful to study gene-centromere recombination rates since the level of recombination is expected to be proportional to any genes distant from the centromere, assuming high interference (Hussain *et al.*, 1994a). It can be used to increase the rate of inbreeding (Thorgaard and Allen, 1987) and useful in gene mapping studies (Kocher *et al.*, 1998).

Due to the complete homozygosity induced by mitotic gynogenesis, this can be applied to produce isogenic fish in the first generation and homozygous clonal lines in the second generation. Gynogenesis can also be used to analyse the genetic basis of complex traits such as sex determination and body colouration. Monosex populations of animals can be produced using this technique by manipulating the sex determining system of the species concern. One of the important applications of gynogenesis is to search for molecular markers for rapid identification of sex in commercially important species of fish with genetic sex determining system.

2.1.2. Microsatellites

The isolation of satellite DNA by buoyant density gradient centrifugation and its characteristic tandemly arranged motifs with highly repetitive DNA sequences was one of the major breakthroughs for the generation of the present day molecular markers (Tautz and Renz, 1984; Epplen *et al.*, 1997). Probably the most powerful Mendelian marker was discovered in 1984 and was termed as microsatellite (Tautz and Renz, 1984; Litt and Luty, 1989; Weber and May, 1989). These are tandemly repeated with a repetitive unit of 1-6 bp. Their variability is most often due to particular arrays on a given chromosome having different repeat numbers in different individuals. Thus, they form allelic variants and for a number microsatellites, almost every individual is heterozygous (Tautz and Renz, 1984; Litt and Luty, 1989; Weber and May, 1989).

Microsatellite sequences are very abundant and well dispersed in the eukaryotic genomes and can account for up to 1% of the genome. They are mainly found within intron regions but can also be found within expressed regions (Edwards *et al.*, 1998). They are also found in prokaryotes (Field and Wills, 1998) and in viruses (Davis *et al.*, 1999). The most commonly studied microsatellites are di- [e.g. (CA)n], tri- [e.g. (CCT)n] and tetra- [e.g. (GATA)n] nucleotide repeats. The arrangement of these repeats in a genome can be pure (e.g. CACACACACACACACA), compound (e.g. CACACACAGAGAGAGAGA) or interrupted (e.g. CACACACAGGGCACACA) (Tautz and Renz, 1984; Litt and Lutty, 1989).

The variability in microsatellites is derived from mutation (Di Rienzo *et al.*, 1994). Two models of mutation have also been proposed to describe variation at microsatellite loci, the infinite allele mutation model (IAM) and the stepwise mutation model (SMM).

The IAM model predicts that mutation will generate only new allelic conditions, which may involve any number of repeat units. In contrast, the SMM model predicts that mutation occurs through the gain or loss of a single repeat unit (reviewed by O'Connell and Wright, 1997). Although the SMM model assumes all changes take place in the repetitive region, allelic size data across species suggests changes also occur in the flanking region (Rico *et al.*, 1996; Angers and Bernatchez, 1997).

2.1.2.1. Abundance of microsatellites in fish genomes

Based on the screening of size-selected genomic libraries from several species, microsatellites appear to be abundant in the genomes of teleost fish. Arrays of (GT)n repeats were reported to occur, on average, every 7 kb in Atlantic cod (Brooker *et al.*, 1994), 12 kb in zebra fish (Goff *et al.*, 1992), 11-56 kb in Atlantic salmon (McConnell *et al.*, 1995a), and 23 kb in brown trout (Estoup *et al.*, 1993). Estoup *et al.* (1993) also screened for (CT)n microsatellites and found that they were about one-third as abundant as (GT)n arrays. The proportion of (GT)n to (CT)n is surprisingly similar to that reported for rats and humans (3:1) (Beckmann and Weber, 1992).

2.1.2.2. Microsatellites and their application in fisheries research

Microsatellites are versatile and almost ideal Mendelian genetic markers for studies of ecology, evolution and conservation (reviewed by O'Reilly and Wright, 1995; Jarne and Lagoda, 1996; O'Connell and Wright, 1997; Neff *et al.*, 2000). These markers have been widely used in many areas of fisheries research, such as to assess the effective population size of stocks (Garcia deLeon *et al.*, 1997; Shaklee and Bentzen, 1998;

Arnegard *et al.*, 1999), stock identification (Shaklee and Bentzen, 1998), levels of inbreeding (Tessier *et al.*, 1997), population structure and gene flow (Garcia deLeon *et al.*, 1997; Arnegard *et al.*, 1999), parentage (Knight *et al.*, 1998; Galbusera *et al.*, 1996, 2000; Jones and Avise, 1997; Peruzzi and Chatain, 2000), quantitative traits (Jackson *et al.*, 1998) and linkage mapping (Kocher *et al.*, 1998; Sakamoto *et al*, 2000).

In Nile tilapia, microsatellites have been used to construct a genetic linkage map (Kocher *et al.*, 1998; McConnell *et al.*, 2000). Phylogenetic relationships have also been studied in several species of the tribe Tilapiini (Sobolewska, 1999). The species composition of tilapia strains has also been investigated using microsatellite loci (Costa-Pierce and Doyle, 1997). Microsatellites have been used in marker-assisted selective breeding of Nile tilapia (Agresti *et al.*, 2000). Polymorphic microsatellite loci have also been used to study paternal transmission in genetically manipulated populations, such as gynogenesis in African catfish (Galbusera *et al.*, 2000) and in European seabass *Dicentrarchus labrax* (Peruzzi and Chatain, 2000). Microsatellites were employed to assign Atlantic salmon families susceptible to infectious pancreatic necrosis virus (Plant, 2000).

Microsatellite loci are usually amplified by PCR as single loci, a process which is time-consuming and not very cost effective. Co-amplification of two or more microsatellite loci in a single PCR, or PCR multiplexing, is a powerful technique, which considerably reduces the time and cost associated with microsatellite genetic assays (Chamberlain *et al.*, 1988; Neff *et al.*, 2000). Since its description (Chamberlain *et al.*, 1988), PCR multiplexing has been successfully applied in many areas of DNA analysis (reviewed by Henegariu *et al.*, 1994). However, optimisation of multiplex PCR is often

difficult and time-consuming. Several protocols for PCR multiplexing using either fluorescent labels with automated detection and on radioisotope labelling have been published (Edwards *et al.*, 1991; Kimpton *et al.*, 1993; Oetting *et al.*, 1995; Paetkau *et al.*, 1995; Henegariu *et al.*, 1997; Ricciardone *et al.*, 1997; Fishback *et al.*, 1999; Neff *et al.*, 2000) and associated problems and solutions have been discussed.

In the research described in this chapter, several second generation clonal lines were propagated from existing clonal lines produced by Sarder *et al.* (1999) and two first generation clonal lines were produced from mitotic gynogenetic females produced by Ezaz (2002). These clonal lines were propagated by meiotic gynogenesis as well as by subsequent sex-reversal of some clonal females to neomales to allow propagation by sib-mating. One outbred clonal line was also produced by crossing females from one clonal line to neomales of another clonal line. The genetic sex of the clonal individuals from all these lines was also tested by progeny testing using suitable test individual. In the present study, attempts were made to use microsatellite loci in multiplex reactions for parentage analysis in clonal as well as normal Nile tilapia. Seven microsatellite loci were screened for heterozygosity (as mentioned in Section 2.2.7.1.) in founder females and donor males used in propagation experiments. To identify the clonal status of the offspring, i.e. elimination of the paternal genome transmission in the clonal progeny and success of female diploidisation, several microsatellite loci with clearly different allele sizes between founder female and donor male were selected and combined in multiplex PCR.

2.2. Material and methods

2.2.1. Fish stocks

The Nile tilapia used in this experiment 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 managed to maintain their genetic variation over the generations, and their overall genetic variation has been studied by McAndrew and Majumdar (1983), Myers *et al.* (1995) and Sodsuk *et al.* (1995).

2.2.2. General management and maintenance of aquarium facilities

The stocking, rearing, nursing and incubating activities were carried out in re-circulating tropical fresh water aquarium facilities at the Institute of Aquaculture, IoA. The temperature of the aquaria was maintained at 27 ± 1°C with a controlled photoperiod of 12 hours of light and 12 hours of dark.

Brood fish were kept in glass aquaria so that maturity states of females could be constantly monitored. Fertilised eggs were incubated in a separate recirculating system fitted with round bottom plastic jars. The water prior to entering into incubation units was treated in a 30 W UV sterilisation unit (flow rate 20 l min^{-1}, UV dosage ca 62000 μW/sec/cm). The water flow rates of incubation jars were controlled by regulatory valves. During the incubation period, eggs were monitored regularly. Dead eggs were removed regularly and survival at different stages of embryonic development was scored.

After hatching and absorption of the yolk sac, the fry were transferred to recirculating fry rearing systems. These systems consisted of a series of 10 l Perspex tanks with a water depth of 13 cm. 50-60 newly hatched fry were stocked in each tank for a period of one month for nursing.

After one month of nursing in the fry rearing system, fry were transferred to re-circulating advanced fry rearing systems comprising a series of 30 l circular plastic tanks with a water depth of 18 cm, 50-60 individuals per tank. They were reared in the circular tanks for up to 3 months, and then transferred to large tanks (2 m diameter) to allow further growth. The basic water quality (such as pH, ammonia, nitrate and nitrite) parameters were checked every fortnight.

Fish of all sizes (from fry to mature broodstock) were fed with an appropriate size (designated as no. 3-5) of commercial trout feed (Trouw Aquaculture Nutrition, UK) at least twice a day. Early fry were fed at least 3 times a day with powdered food (0.25-1.0 mm in diameter) prepared by grinding no. 3 size feed in a coffee grinder. As the fish grew the feed size was increased. Advanced fry and fingerlings, weighing between 10 g and 40 g, received number 3-sized feed at a rate of 3-7.5% of body weight at least twice a day. Fish weighing 40 g - 80 g and 80 g and above were fed with number 4 and number 5-sized feed respectively, at a rate of 2-3% of their body weight per day.

To allow breeding to be monitored and to allow fish from different families to be kept in the same stock tank, fish were tagged at 3-4 months old with Passive Integrated Transponder (PIT) tags (Trovan, Inc., USA or Avid Inc., USA). Each tag contains an individual code (Trovan tag -10 digit code; Avid tag - 9 digits code) that can be read by a specific tag reader. This small tag was placed into the body cavity of the fish through

a small lateral incision made just above the anus with a sharp sterile scalpel blade. After insertion of the tag, Surgical adhesive (Orahesive protective powder, Squibb and Sons Ltd., UK.) was spread over the incision to assist adhesion and to prevent infection. A proprietary tagging syringe with a sharp specially designed needle was also used to tag larger fish.

Fish were anaesthetised to avoid excessive handling stress prior to breeding, tagging and sampling (blood collection, fin clipping). For this purpose benzocaine (ethyl 4-aminobenzoate) solution at a final concentration of 1: 10,000 was used as an anaesthetic. A stock solution was first prepared by dissolving benzocaine powder at 10% (w/v) in ethanol.

All procedures of fish breeding, anaesthesia, tagging, sampling and genetic manipulation were performed under project and personal licences issued by the U.K. Home Office.

2.2.3. Production and propagation of clonal lines

2.2.3.1. Gynogenesis

Initially meiotic gynogenesis was performed for the production and propagation of clonal lines.

2.2.3.1.1. Fish stocks

Fish used for the production and propagation of homozygous and heterozygous clonal lines in the present experiment came from two different sets of experiments conducted at the Institute of Aquaculture (Table 2.1) (Sardar *et al.*, 1999; Ezaz, 2002).

Table 2.1. List of fish used in the production and propagation of clonal lines.

PIT Tag Number	Sex		Other		Reference
	Phenotype	Genotype	Phenotype	Genotype	
010 891 117	♀	XX	Blond	blbl	Clone A (homozygous clone)
010 024 341	♀	XX	Blond	blbl	Clone A (homozygous clone)
005 299 334	♀	XX	Blond	blbl	Clone B (homozygous clone)
007 298 637	♀	XX	Blond	blbl	Clone C (homozygous clone)
008 377 098	♀	XX	Blond	blbl	Clone C (homozygous clone)
008 618 807	♀	XX	Blond	blbl	Clone C (homozygous clone)
00 013C A861	♀	XY	Red	RR	Mitotic gynogen (founder of homozygous clonal line D)
00 0135 BE59	♀	XY	Red	RR	Mitotic gynogen (founder of homozygous clonal line E)
00 0610 906F	♀	XX	Red	RR	Clone D (homozygous clone)
00 0610 9341	♀	XX	Red	RR	Clone D (homozygous clone)
00 0610 8F94	♀	XX	Red	RR	Clone D (homozygous clone)
00 0610 9519	♀	XX	Red	RR	Clone D (homozygous clone)
00 0610 848F	♀	XX	Red	RR	Clone D (homozygous clone)
00 0610 8477	♀	XX	Red	RR	Clone D (homozygous clone)
00 0610 978F	♀	XX	Red	RR	Clone E (homozygous clone)
00 0610 9BBA	♀	XX	Red	RR	Clone E (homozygous clone)
00 0610 685D	♀	XX	Red	RR	Clone E (homozygous clone)
00 0610 6520	♀	XX	Red	RR	Clone E (homozygous clone)
00 0610 7822	♀	XX	Red	RR	Clone E (homozygous clone)
00 0610 8D77	♀	XX	Red	RR	Clone E (homozygous clone)
014 556 527	♂	YY	Wildtype	rr	MT-treated (donor male)
005 117 817	♂	XX	Wildtype	rr	MT-treated (donor male)
00 0610 5834	♂	XX	Blond	blbl	Clone A (homozygous clonal neomale)
00 0610 8554	♂	XX	Blond	blbl	Clone B (homozygous clonal neomale)
00 0610 69A3	♂	XX	Blond	blbl	Clone B (homozygous clonal neomale)
00 0610 9D05	♂	XX	Red	RR	Clone D (homozygous clonal neomale)
00 0610 7982	♂	XX	Red	RR	Clone D (homozygous clonal neomale)
00 0610 7991	♂	XX	Red	RR	Clone E (homozygous clonal neomale)
00 0610 7EE7	♂	XX	Red	RR	Clone E (homozygous clonal neomale)

2.2.3.1.2. Collection and ultraviolet (UV) irradiation of milt

Milt was collected from the male by hand stripping. Before stripping, faeces and mucus were cleaned off the urogenital papilla by using a piece of soft tissue. Hand stripping involves application of gentle downward pressure with the thumb and index fingers from below the pectoral fin to the genital opening of the fish. The milt was collected in a glass capillary tube by placing it at the opening of the urethra. Milt contaminated with urine was discarded. Milt was kept at 4°C until use. Sperm used for gynogenesis were first checked for motility under a microscope and then the sperm concentration was determined using a haemacytometer. For this, milt was diluted 500

times in modified fish Ringers (MFR) solution, pH 8.0 (MFR; 112 mM NaCl; 68 mM KCl; 2.5 mM $NaHCO_3$; 15 mM $CaCl_2.6H_2O$) by adding 10 µl of milt to 490 µl of MFR in a 1.5 ml tube to make the total volume of 500 µl and mixed by gentle shaking, then 10 µl of diluted milt from the first tube was placed into another 1.5 ml tube 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 cover slip. When the sperm settled down after a few minutes, the number of sperm in five large squares (each subdivided into 16 small squares) was determined and the sperm concentration calculated as described by Sarder *et al.* (1999). For example:

- total number of sperm in five large squares on one side was 200 and on the other side was 250
- average number of sperm in five large squares = (200+250)/2 = 225
- average number of sperm in a small square = 225/80 = 2.81
- total concentration of sperm = $2.81 \times \frac{4000 \times 1000 \times}{\text{chamber volume}} \quad \frac{50 \times 10}{\text{dilution factor}} = 5.6 \times 10^9 \text{ ml}^{-1}$

For UV irradiation the number of sperm was adjusted to 2.5×10^7 ml^{-1} (optimum, Hussain *et al.*, 1993) by diluting 1000. $(2.5 \times 10^7)/ (5.8 \times 10^9) = 4.31$ µl of dry sperm with MFR to give the total volume 1000 µl.

Diluted milt was transferred to a 30 mm diameter Petri dish for UV irradiation, where the depth of the diluted sperm solution was approximately 1.2 mm. The bottom of this Petri dish had previously been scoured with fine sand (Sigma, UK) to remove its hydrophobicity and to allow the sperm suspension to spread evenly across the Petri dish. The UV irradiation was carried out at 4°C using a 254 nm wavelength UV lamp (Ultra-Violet Products, San Gabriel, California) at a dose of 250-260 µW cm^{-2} for 2 m with gentle stirring using a mechanical shaker. Before irradiation, the intensity of the UV

irradiation was measured by a radiometer (Ultra-Violet Products, San Gabriel, California).

2.2.3.1.3. Spawning females

Mature females of Nile tilapia normally spawn at intervals of approximately 2-3 weeks under aquarium conditions. Mature females can normally be identified by their swollen urogenital papilla and rounded abdomen and sometimes by pre-spawning behaviour (such as gulping at the tank bottom – indicative of nest building and cleaning). The eggs were collected from the mature females by hand stripping. Eggs were collected in a clean plastic Petri dish (100 mm in diameter) and were washed carefully with water from the recirculatory system several times to get rid of any blood and ovarian fluid.

2.2.3.1.4. Fertilisation and heat shock

Eggs were divided into three batches. The first batch (containing approximately 50-100 eggs) was fertilised with UV irradiated milt and the second batch (containing approximately 100 eggs) with normal sperm. The first and second batches were transferred to the incubation unit as UV control and normal control respectively. The third batch (containing approximately 500-1000 eggs) was fertilised with UV irradiated sperm and used to produce meiotic gynogenetic individuals by applying a heat shock.

The heat shock method followed here was as described by Sarder (1998) with minor modifications. Eggs were fertilised *in vitro* with UV treated sperm in a clean Petri dish. Fertilised eggs were kept at room temperature (approx. 20°C) on bench for 4 m allowing

them to proceed towards the second meiotic division. At 4 min post fertilisation, the eggs were carefully transferred to a strainer placed on a water bath (Jencons Scientific Limited, England), set at 42° C. The heat shock was applied for 4 min duration to induce diploidisation by preventing extrusion of the second polar body. After the heat shock period was over, the strainer with the eggs was removed immediately back to another water bath set at 28°C for few min to allow acclimatisation, and finally transferred to the incubator.

2.2.4. Sex reversal

Masculinisation of XX females was performed to produce XX neomales (genotypically female but phenotypically male; Popma and Green, 1990), by feeding hormone treated feed starting from first feeding. Usually half of the fry of a batch were fed twice in a day with hormone treated food from 9-10 days post fertilisation for 30 consecutive days followed by untreated (normal) feed. The remaining half was kept as untreated control to give the original sex ratio.

The hormone used for this purpose was 17 α-methyl testosterone at 60 mg kg^{-1} of food. To prepare the feed, number 3 trout food pellets were ground to give a fine powder and thinly spread out in a tray. The required amount of hormone (17 α-methyl testosterone) was weighted and dissolved in absolute ethanol. The dissolved hormone was sprayed over the food in a fume cupboard using a spray gun (BDH) and the food was 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 group fry was prepared in the same way by spraying only absolute ethanol.

2.2.5. Sexing tilapia

Two sexing methods were used. For large, mature fish, external examination of the urogenital openings could easily reveal the sex. The male has a single urinary and genital opening, whereas the female has separate openings for the urinary tract and oviducal canal (Chervinski, 1983). The second method was more accurate but involved dissection and examination of gonads, which required the fish to be killed (Guerrero and Shelton, 1974). The aceto-carmine method is only used for small fish (i.e. too small for sexing on the basis of the papilla). In this method, each fish was killed by an overdose of benzocaine and subsequent destruction of the brain. The gonads were then dissected out on to clean glass slide and squashed with a drop of aceto-carmine stain by a cover slip. The sex of fish could then be easily revealed by examining the squashed gonads under 500 magnification using a compound microscope. Although this method required the fish to be killed, it is quick, accurate and can be used to sex fish as small as 25 mm in standard length (7-8 weeks old).

2.2.5.1. Experimental plan for the production of homozygous and heterozygous clonal lines

Propagation of clonal lines derived from the experiments of Sarder *et al.* (1999) involved meiotic gynogenesis using the eggs of the existing clonal fish from three different clonal lines (see reference from Table 2.1). At the same time several batches of clonal fish from these three clonal lines were also sex reversed to neomales in order to propagate these three clonal lines by sib mating (Fig. 2.1).

For the other two clonal lines, mitotic gynogentic females were collected from the experiment conducted by Ezaz (2002) (see reference from Table 2.1). Another round of

meiotic gynogenesis was performed in order to produce clonal lines from these two females. In addition several batches of clonal fish were also sex reversed into neomales in order to propagate clonal lines by sib mating (Fig. 2.2). To produce outbred clonal line (OBC), females from clonal line A were crossed to neomales from clonal line B (Fig. 2.3).

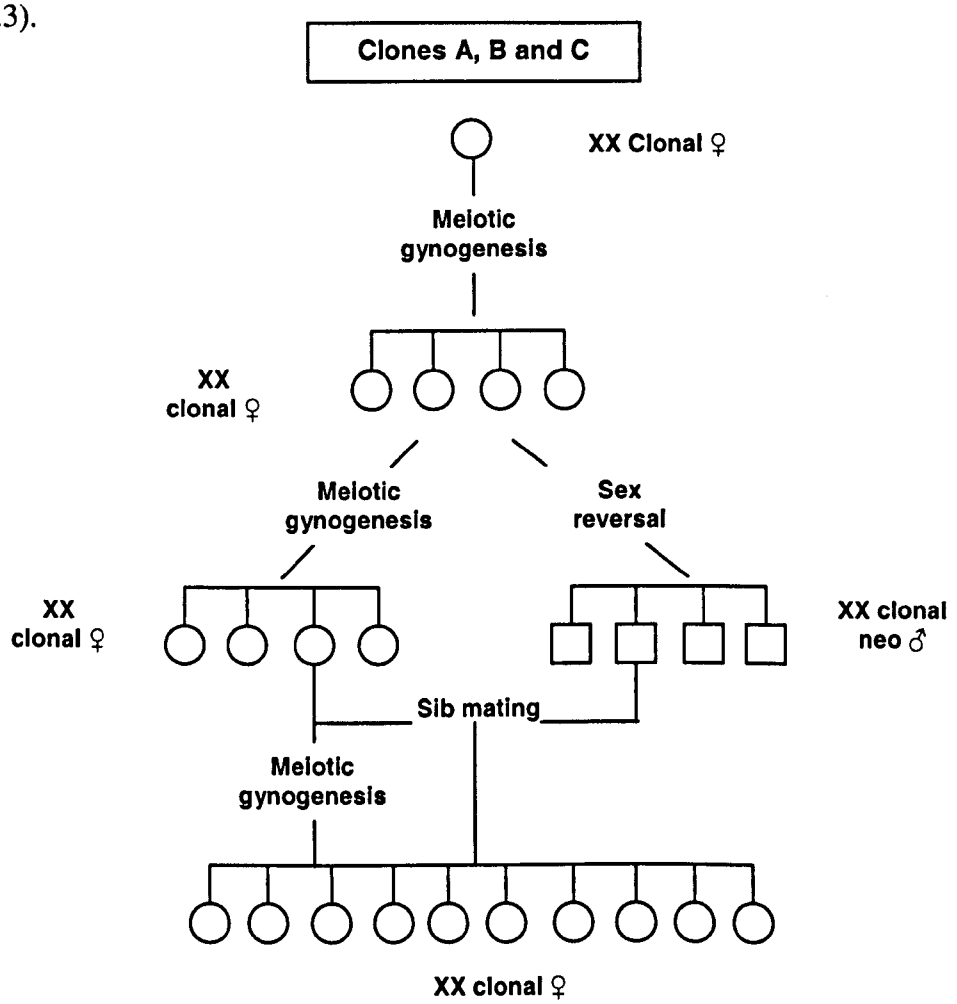


Fig. 2.1. Schematic diagram of propagation of *Oreochromis niloticus* clonal lines in A, B and C.

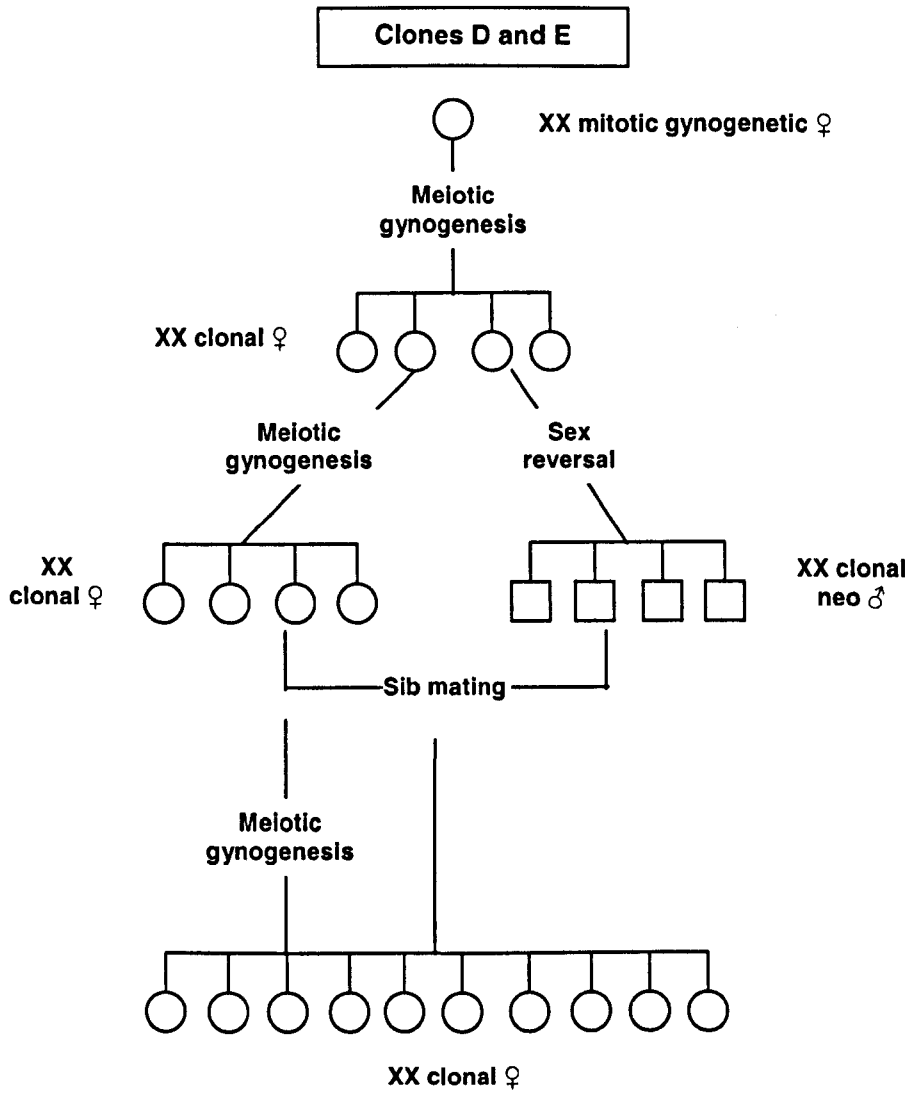


Fig. 2.2. Schematic diagaram of production and propagation of *Oreochromis niloticus* in clonal lines D and E.

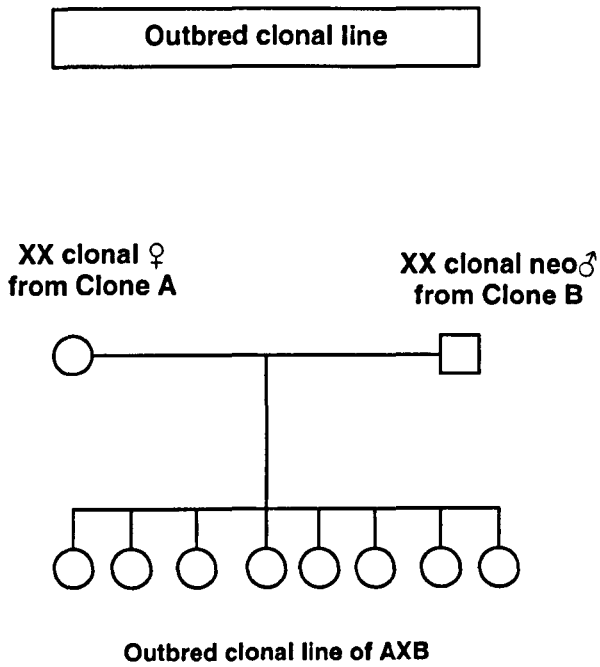


Fig. 2.3. Schematic diagram of production of outbred clonal line (OBC) in *Oreochromis niloticus* by crossing clonal lines A and B.

2.2.6. DNA extraction

2.2.6.1. Sample collection

Both fin and blood samples were collected for DNA extraction. Blood samples were collected from the caudal vein of fish using 1 ml syringes with 25GX5⁄8″ to 23GX1″ needles. Modified Cortland's saline (124 mM NaCl; 1.1 mM $CaCl_2.2H_2O$; 2.6 mM NaH_2PO_4; 12 mM $NaHCO_3$; 2 mM $MgSO_4$ and 5.6 mM Glucose) was used with 10 mM EDTA added as an anticoagulant. Approximately one volume of Cortland's saline and two volumes of blood were taken in the syringe. The collected blood was transferred into a labelled 1.5 ml sterile tube and centrifuged at 1500g for 4 min in a bench top centrifuge. After centrifugation, the supernatant was removed and the precipitated red blood cells were immediately immersed in a flask containing liquid nitrogen. The

frozen blood cells were stored at -20°C until required. Fin samples were collected by clipping around 3 mm of tissue from one corner of the caudal fin using sterile scissors. Fin samples were stored in absolute ethanol at 4°C.

2.2.6.2. Extraction of total genomic DNA

Total genomic DNA was extracted from both blood and fin tissues. Ten µl of blood or approximately 50 mg of fin tissue were used. Fin tissue was minced using small, sharp scissors. Minced fin, fresh or thawed blood was transferred into sterile 2 ml screw cap tubes containing 435 µl of TEN buffer (100 mM Tris-HCl, pH 8.0; 10 mM EDTA; 250 mM NaCl), 10 µl of 20 mg ml^{-1} Proteinase K (Kramel Biotech, UK) and 50 µl of 10% (w/v) SDS (sodium lauryl sulphate, Sigma, Dorset, UK) solution. In the case of blood, the SDS was added after dispersion of the sample in buffer. The mixture was mixed gently and incubated at 55°C in a rotating oven overnight (6-18 h).

After incubation, 10 µl of 20 mg ml^{-1} DNAse free RNAse (RNAse A; Sigma, UK) was added to the mixture, which was incubated at 37°C for 1 hr. After 1 hr of incubation, 500 µl of buffered phenol was added to the mixture, which was mixed at room temperature for 15-20 m. The mixture was then centrifuged at 16,000g for 12 min and the supernatant transferred to another sterile tube. This step was repeated once and 500 µl of 24:1 (v/v) chloroform:isoamyl alcohol was then added to the solution and mixed at room temperature for 15-20 min. This step was repeated once. The mixture was then centrifuged again as described previously and the supernatant transferred to another sterile tube. Then 0.6-0.8 volume of chilled isopropanol was added to the

solution, which was shaken vigorously for approximately one minute to precipitate the DNA.

The DNA pellet was then transferred to another sterile tube containing 500-700 µl of 70% ethanol, using a sterile pipette tip. The pellet was then washed twice in 70% ethanol. Finally, the ethanol was discarded and the pellet was dried and resuspended in 100 µl of $TE_{0.1}$ (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA) at 45-50°C for 30 min, with total re-suspension taking several days or weeks. The resuspended DNA solution was kept at 4°C for immediate use or frozen at -20°C for long-term storage.

2.2.6.3. Quality and quantity of DNA

The quality of DNA was checked by electrophoresis on a 0.7% agarose gel, using 0.5X TAE (1 l of 50X solution contain 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5M EDTA, pH 8.0) running buffer, for 3-4 hours at a constant 5V cm^{-1}. The gel was stained with ethidium bromide (10 mg ml^{-1}) at a rate of 5 mg/100 ml and visualised using a UV transilluminator.

The quantity of DNA was determined by comparing the ratio of absorbance at 260 and 280 nm in a spectrophotometer. The quantity of DNA was also assessed by agarose gel electrophoresis of high molecular weight DNA with a standard DNA size marker.

2.2.7. Microsatellite genotyping

2.2.7.1. Microsatellite primers

Seven di-nucleotide repeat microsatellite loci were selected from those isolated from Nile tilapia (Lee and Kocher, 1996). Primers were selected mainly on the basis of degree of heterozygosity. Four of those (UNH189, UNH197, UNH203 and UNH208) were found informative in an earlier experiment on the phylogenetics of the Tilapiines at the Institute of Aquaculture (Sobolewska, 1999). The heterozygosity information about the other loci (UNH127, UNH211 and UNH228) was kindly provided by Professor Tom Kocher (personal communication).

Table 2.2. Microsatellite primers used in the parentage analysis.

Loci name	Primer sequences and labelling			%	Allele size	EMBL
	Forward primer 5' - 3'	ABI dye	Reverse primer 5' - 3'	heterozygosity	range (bp)	accession no.
UNH127	TCTAATGGTTCTTATTATCTCA	6-FAM	CTATACATGATTGTGCAATAAATAA	100	126-128	G12280
UNH189	ATCGATGCTTTAAGAATCAG	6-FAM	TTCTCTGACATTTTTCAGC	73	144-181	G12341
UNH197	CAGGATGGTGAGATGTTT	HEX	TTAAGTGGAAGAAGTCAATG	62	158-209	G12348
UNH203	CACAAAGATGTCTAAACATGT	TET	GAATTTGACAGTTTGTTGTTTAC	95	86-107	G12354
UNH208	CTTCTTGGCCTACAATTT	6-FAM	CAGATGGGTGATAGCAA	77	86-106	G12359
UNH211	GGGAGGTGCTAGTCATA	TET	CAAGGAAAACAATGGTGATA	100	130-160	G12362
UNH228	ACACCTTCACACAACTACG	TET	GATAATAATGATAATGTGGCCT	100	214-216	G12379

All primers were synthesised at 0.01 μmol scale by MWG-Biotech (UK Ltd.). The forward primers were labelled at the 5′ end with three different ABI fluorescent dyes (FAM, HEX and TET) for fragment analysis in an ABI PRISM™ 377 DNA sequencer (PE Applied Biosystems). The forward primers were labelled with ABI fluorescent dyes on the basis of the allele size range. Primers with overlapping allele size were labelled with different coloured dyes to avoid ambiguity during data analysis (Table 2.2; Fig. 2.4). The forward primers were diluted in $TE_{0.1}$ to 100 μM stock and aliquoted as 10 μM working solutions.

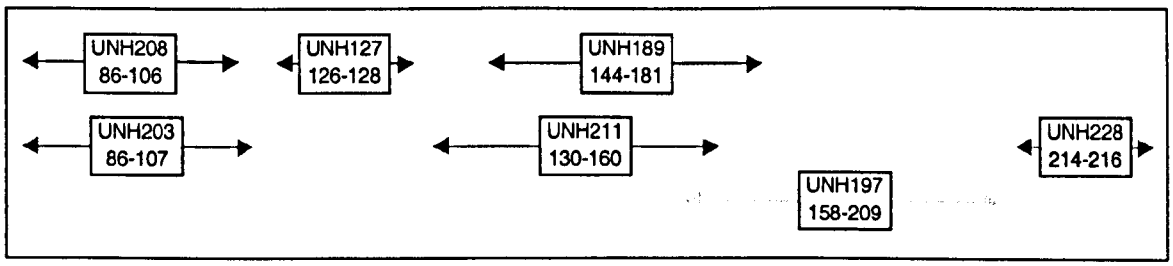


Fig. 2.4. Schematic representation of allele size range and ABI fluorescent dye labelled microsatellite loci used in the present experiment (after Ezaz, 2002).

Reverse primers were resuspended in $TE_{0.1}$ (10 Mm Tris, pH 8.0; 0.1 mM EDTA) to 100 µM stock solution and aliquoted into several sterile tubes as 10 µM working solutions. Both stock and working primer solutions were stored at -20°C. ABI dye labelled forward primers were kept in dark vials as well as boxes, and shielded from excessive light to prevent photoactive degradation.

2.2.7.2. Single locus PCR optimisation for microsatellite loci

All seven microsatellite primer pairs were first optimised as single locus PCRs. A temperature gradient PCR was performed (T gradient thermocycler; Whatman Biometra) to optimise the PCR conditions for ten different annealing temperatures and three different $MgCl_2$ concentrations.

Single locus PCR was performed in 10 µl reaction volume containing 1X PCR buffer, 1.0, 1.5 or 2.0 mM $MgCl_2$, 200 µM of dNTPs, 0.75 µM of each primer and 0.5 units of *Taq* DNA polymerase (Advanced Biotechnologies, UK) and 40-60 ng of DNA template. All seven loci were optimised separately first in two randomly selected DNA samples.

The gradient PCR conditions included an initial denaturation at 96°C for 3 min, then 5 cycles comprising denaturation at 95°C for 50 sec, annealing at ten temperatures (temperature at each vertical lane of PCR block were: 49.5°C, 50.9°C, 53.0°C, 55.4°C, 57.8°C, 60.2°C, 62.6°C, 64.9°C, 67.1°C and 68.5°C respectively) for 50 sec and extension at 72°C for 1min 10 sec, then 26 cycles of PCR were performed with the same parameters as the earlier 5 cycles except that the initial denaturation temperature was reduced to 94°C instead of 95°C. Finally an extension of 7 and 30 min at 72°C and 60°C respectively was performed.

After gradient PCR, amplified products were electrophoresed on 1.5% agarose gel with 0.5X TAE running buffer. Successfully amplified products were analysed on a 5% Long Ranger® (Biowhittaker Molecular Applications, ME, USA) gel using an ABI PRISM™ 377 DNA sequencer.

Once single locus PCR was optimised for all seven loci, another round of single locus PCR was performed on a panel of broodstock, comprising all founder females and the donor males used in this experiment. The results of single locus PCR on the broodstock were analysed and a multiplex PCR plan was designed on the basis of allelic variation between the founder females and the donor males.

2.2.7.3. Multiplex PCR optimisation for microsatellite loci

On the basis of single locus analysis, two multiplexed PCRs involving five and three loci respectively were designed for five clonal lines of Nile tilapia using a touchdown PCR programme. For parentage assignment in each clonal line a sample set of 12 individuals were analysed, comprising founder female, donor male, six randomly

selected clonal and four randomly selected control individuals.

A reaction volume of 10 µl PCR was prepared as described in Section 2.2.7.2 except that the primer concentrations were: 0.4 µM of each primer for the duplex reaction, 0.3 µM of each primer for the triplex reaction and 0.2 µM of each primer for the pentaplex reaction.

The touchdown PCR programme designed for this purpose included an initial denaturation at 96°C for 3 min, 10 cycles of denaturation at 95°C for 50 sec, annealing at 60°C with 0.5°C decrease in each cycle for 50 sec and extension at 72°C for 3 min, then 25 cycles with denaturation at 94°C for 50 sec, annealing at 55°C for 50 sec and extension at 72°C for 3 min; and a final extension at 72°C and 60°C for 7 min and 60 min respectively.

The PCR products were first electrophoresed on 2% agarose gels with 0.5X TAE running buffer, before running on 5% LongRanger® gel in an ABI PRISM™ 377 DNA sequencer for fragment analysis.

2.2.7.4. Fragment analysis on ABI automated DNA sequencer and data analysis

Electrophoresis of PCR amplified microsatellite loci was performed on an ABI Prism™ 377 DNA sequencer according to the manufacturer's instructions (GeneScan™ Analysis software Users Manual, The PE Corporation).

A 0.2 mm thick 5% denaturing LongRanger® (Biowhittaker Molecular Applications, ME, USA) gel was prepared with 1X TBE running buffer. First, the glass plates (36 cm well to read distance) were washed in warm water and rinsed with distilled water. Plates were then air dried completely before pouring the gel solution. A 50 ml gel mix was

prepared by mixing 18 g urea, 26 ml double distilled water, 5 ml of 50% LongRanger® solution and 0.5 g mixed bed resin. The gel mixture was stirred for half an hour (to bring it to the room temperature) and filtered through a 0.20 µm filter. Five ml of 10X TBE buffer was also added during filtration. The filtered gel mixture was degassed by exposure to vacuum for 4 m. After de-gassing, 250 µl of freshly prepared 10% ammonium persulfate (APS) and 25 µl of N,N,N′,N′-Tetramethylethylenediamine (TEMED, Sigma, UK) was added to the gel solution, which was mixed and poured carefully using a 50 ml syringe. The gel was then left for 2-2.5 hours to allow polymerisation.

For fragment analysis of microsatellite loci in ABI automated sequencer, 1 µl of a ten-fold diluted amplified PCR products was mixed with 1 µl of loading buffer mix (containing deionized formamide, 50 mg ml^{-1} blue dextran with 25 mM EDTA (pH 8.0) and GeneScan™-350 [TAMRA; PE Applied Biosystems] size standard in a ratio of 5:1:2).

After electrophoresis in the ABI automated sequencer, the gel image was tracked, the appropriate matrix standard was installed and the lanes were extracted according to the instruction manual. After extraction, analysis parameters were set and data were analysed using GeneScan™ (V. 2.1) and Genotyper™ (V. 2.1) analysis software (PE Corporation).

2.2.8. Statistical analysis

Heterogeneity chi-square analysis was performed on pooled survival rates data. Survival rates at different stages of development were compared by ANOVA (General

linear model with Bonferroni pair-wise multiple comparison tests) with Minitab (V.13) statistical analysis software using arcsine transformation of percent data.

2.3. Results

2.3.1. Survival rates of homozygous and outbred clonal lines and control groups of *O. niloticus*

The survival rates of homozygous and outbred clonal (OBC AXB) lines and their control groups are presented in Table 2.3.

Survival of both pigmentation and yolk sac absorption (YSA) stages in all clonal lines produced by meiotic gynogenesis was significantly ($P<0.05$) lower than the same stages of clonal lines produced by sib-mating as well as their corresponding control groups.

Table 2.3. Percent survival (% mean ± SE) of homozygous and outbred clonal lines and corresponding control groups of *Oreochromis niloticus*; DP= did not produce; RC= relative to control.

Clonal lines	Developmental stages	Survival rates (% mean ± SE)							
		Control		Clone (Meiogyne)[a]		Clone (Sibmating)		Outbred clone (AXB)	
		Number	% mean ± SE	Number	% mean ± SE	Number	% mean ± SE	Number	% mean ± SE
A	eggs fertilised	1100		9136		3907		4064	
	Pigmentation	791	71.91 ± 1.69	1795	19.49 ± 1.02*	3047	78.95 ± 2.34	3239	79.48 ± 1.60**
	YSA	596	54.18 ± 1.72	364	4.08 ± 0.42	6954	65.71 ± 4.02	2833	70.19 ± 2.01**
B	eggs fertilised	1000		7567		3301		DP	DP
	Pigmentation	719	71.90 ± 2.55	1172	15.25 ± 1.16	2079	64.20 ± 3.71	DP	DP
	YSA	515	51.50 ± 2.58	385	5.09 ± 0.45	1786	55.40 ± 2.99	DP	DP
C	eggs fertilised	2900		18166		DP	DP	DP	DP
	Pigmentation	1350	46.55 ± 2.26	1006	5.42 ± 0.60	DP	DP	DP	DP
	YSA	864	29.79 ± 1.74	134	0.68 ± 0.13	DP	DP	DP	DP
D	eggs fertilised	1000		5405		2449			
	Pigmentation	522	52.20 ± 5.16	287	4.78 ± 1.26	857	35.72 ± 2.01	DP	DP
	YSA	294	29.40 ± 4.52	143	2.30 ± 0.77	514	21.64 ± 2.05	DP	DP
E	eggs fertilised	900		4918		2520			
	Pigmentation	473	52.56 ± 6.62	358	7.06 ± 2.18	756	30.78 ± 1.46	DP	DP
	YSA	235	26.11 ± 4.06	138	2.70 ± 0.81	430	17.64 ± 1.19	DP	DP

[a] Significantly different from both corresponding control and clonal lines produced by sib-mating
* significantly different from YSA
**significantly different from survival in both stages of clonal line A and B produced by meiotic gynogenesis

No significant difference was observed in survival at pigmentation and YSA stage among the clonal lines produced by meiotic gynogenesis. A significant ($P<0.05$) drop in

survival from pigmentation to YSA stage was observed only in clonal line A. Significantly ($P<0.05$) higher survival at both pigmentation and YSA was observed in OBC (AXB) compared to both clonal line A and clonal line B produced by meiotic gynogenesis. No significant difference in survival was observed at either developmental stages between OBC (AXB) and clonal line A and clonal line B produced by sib-mating.

2.3.2. Sex ratios of homozygous and outbred clonal lines and control groups of Nile tilapia

The sex ratios of four homozygous clonal lines and one outbred clonal (AXB) line are summarised in Table 2.4. Sex of adult clonal fish from all four clonal lines were first verified by examining the genital papilla. No males were observed in any of the clonal lines. Then a group of randomly selected individuals from each clonal line was verified by progeny testing by crossing with a YY supermale or XX neomale (mentioned as control group). The results from the control crosses showed that individuals progeny tested from clonal lines A and B produced all male progeny when crossed with a YY supermale and clonal lines D and E produced all female progeny when crossed with a XX neomale (Table 2.4).

Table 2.4. Sex ratios of homozygous and outbred clonal lines and control group of *Oreochromis niloticus*. Sex ratios are presented as female:male (F:M) with percentage of males in parentheses; DP= did not produce.

Clonal lines	Clone (meiogyne)		Clone (sib mating)		Outbred clone (AXB)		Control		Males used to produce control fish
	No. of fish sexed	Sex ratio (%) ♀:♂ (%♂)	No. of fish sexed	Sex ratio (%) ♀:♂ (%♂)	No. of fish sexed	Sex ratio (%) ♀:♂ (%♂)	No. of fish sexed	Sex ratio (%) ♀:♂ (%♂)	
A	100	100:0 (0)	100	100:0 (0)	100	100:0 (0)	57	0:100 (100)	YY male
B	100	100:0 (0)	100	100:0 (0)	100	DP	62	0:100 (100)	YY male
D	100	100:0 (0)	100	100:0 (0)	100	DP	76	100:0 (0)	XX neomale
E	100	100:0 (0)	100	100:0 (0)	100	DP	49	100:0 (0)	XX neomale

Similarly, adult fish from the outbred clonal line (OBC AXB) were also examined by their genital papilla and all found to be females. Randomly selected individuals form OBC (AXB) were also progeny tested with the same YY supermale. Progeny of these crosses were all males (Table 2.4).

2.3.3. Verification of clonal status using microsatellite loci

2.3.3.1. Single locus PCR optimisation

The results of single locus optimisation with 1.5 mM $MgCl_2$ concentration are summarized in Table 2.5. This revealed PCR amplified products in a range of different temperatures at all loci.

Table. 2.5. Single locus annealing temperature optimisation of all seven loci with 1.5 mM $MgCl_2$ concentration.

Loci	Annealing temperature (gradient)									
	49.5	50.9	53.0	55.4	57.8	60.2	62.6	64.9	67.1	68.5
UNH127	+	+	+*	+	+	-	-	-	-	-
UNH189	+	+	+	+*	-	-	-	-	-	-
UNH197	+	+	+	+*	+	-	-	-	-	-
UNH203	+	+	+	+	+*	-	-	-	-	-
UNH208	+	+	+	+*	-	-	-	-	-	-
UNH211	+	+	+	+	+*	+	-	-	-	-
UNH228	+	+	+	+*	+	-	-	-	-	-

+: PCR amplification; -: no amplification; * optimised annealing temperature based on GeneScan™ fragment analysis.

The annealing temperature for each locus was selected on the basis of fragment analysis by electrophoresis on 5% denaturing LongRanger® gel using an ABI automated DNA sequencer, with subsequent data analysis by GeneScan™ (V. 2.1) and Genotyper™ (V. 2.1) analysis software (PE corporation). The annealing temperatures that generated the most clearly defined bands with the fewest stutter bands were selected

for later PCR amplification. Based on the Genotyper™ and GeneScan™ analysis, 55°C annealing temperature was selected for loci UNH189, UNH197, UNH208 and UNH228, 53°C for locus UNH127 and 58°C for loci UNH203 and UNH211 (Table 2.5).

Considering the $MgCl_2$ concentrations, concentrations of 1.0 and 1.5 mM appeared to be best for all loci with fewer false and stutter bands in comparison to PCR reactions with 2 mM $MgCl_2$ concentration. Moreover, PCR reactions using 2.0 mM concentration required a higher annealing temperature than those with 1.0 and 1.5 mM concentrations (Fig. 2.5). Therefore, 1.5 mM $MgCl_2$ concentration was selected for this experiment.

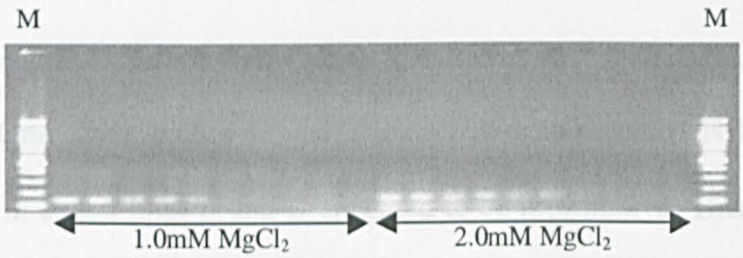


Fig. 2.5. Single locus optimisation of locus UNH127 at ten different annealing temperatures (49-69°C, left to right) and two different $MgCl_2$ concentrations (1.0 mM and 2.0 mM) for one DNA sample. M: 100 bp ladder DNA size marker.

The results of the single locus screening of the brood stock at all seven microsatellite loci are presented in Tables 2.6 and 2.7. Five founder females of clonal lines and two donor males were screened for all seven loci optimised earlier in two DNA samples. The allelic variation and heterozygosity in all founder females were analysed and compared to the corresponding donor male to find suitable loci for parentage analysis (Table 2.6 and 2.7).

Table 2.6. Alleleic variation of seven microsatellite loci in broodstocks used for production and propagation of clonal lines in *O. niloticus* reveled from single locus analysis. * last three/four digits of PIT tag numbers. Letters A, B, C, D and E refers to the founder female of clonal lines; ♂ (527): YY donor supermale and ♂ (817) XX donor neomale.

Loci	Fish PIT tag numbers* and allele size (bp)						
	A (117)	B (334)	C (094)	D (A861)	E (BE59)	♂(527)	♂ (817)
UNH 127	128/128	124/124	130/130	124/124	124/124	130/130	126/128
UNH 189	153/153	153/153	153/153	168/168	168/168	158/168	153/178
UNH 197	166/166	156/156	166/166	190/190	190/190	181/181	166/166
UNH203	89/89	85/85	89/89	83/83	83/83	85/101	83/89
UNH 208	103/103	86/86	84/84	100/100	111/111	86/100	93/103
UNH 211	126/126	129/129	136/136	133/133	136/136	117/131	126/136
UNH228	228/228	228/228	228/228	214/214	214/214	224/228	214/224

Based on the evidence of allelic variation data between the founder females and the donor male, five microsatellite loci (UNH 189, UNH 203, UNH 208, UNH 211 and UNH 228) in clonal lines A, B and E and three microsatellite loci (UNH 189, UNH 208 and UNH 228) in clonal lines C and D appeared to be informative. These microsatellite loci were selected for parentage analysis in the five clonal lines, as single loci or multiplexed PCR (Table 2.7).

The heterozygosity and the number of alleles produced by all the seven loci screened on seven individuals are summarized in Table 2.7. The observed heterozygosity at these seven loci ranged from 0-29%. This is because except for the two donor males, three founder females were clonal and two founder females were mitotic gynogenetics in origin. The number of alleles observed per locus ranged from 3-6.

Table 2.7. Summarized results for single locus broodstock screening with seven microsatellite loci. Published allele size ranges are in parentheses.

Loci	Number of individuals	Number of alleles	Observed allele size range (bp)	Number of heterozygotes*	% heterozygosity
UNH127	7	4	124-130 (126-128)	1	14.29
UNH189	7	4	153-178 (144-181)	2	28.57
UNH197	7	4	156-190 (158-209)	0	0.00
UNH203	7	4	83-101 (86-107)	2	28.57
UNH208	7	6	84-111 (86-106)	2	28.57
UNH211	7	6	126-136 (130-160)	2	28.57
UNH228	7	3	214-228 (214-216)	2	28.57

* only the two donor males were expected to be heterozygous for any loci

2.3.3.2. Multiplex PCR for microsatellite loci

The results of the multiplex PCR at microsatellite loci are presented in Table 2.8. As mentioned in the methodology (section 2.2.7.3) pentaplex PCR for clonal lines A, B and E (loci UNH 189, UNH203, UNH 208, UNH 211 and UNH 228) and triplex PCR for clonal lines C and D (loci UNH 189, UNH 208 and UNH 228) were attempted. The results are summarized in Table 2.8 and an example of a GeneScan™ (V. 2.1) electropherogram is presented in Fig. 2.6.

Table 2.8. Microsatellite multiplex in five clonal lines of *O. niloticus*. *: successful in PCR multiplexing.

Loci	Clonal line	Sample set											
		Founder	Donor	Gynogenetic Clones						Controls			
		female	male	1	2	3	4	5	6	1	2	3	4
UNH 189*	A (117 X 527)	153/153	158/168	153/153	153/153	153/153	153/153	153/153	153/153	153/158	153/168	153/168	153/168
UNH 203*		89/89	85/101	89/89	89/89	89/89	89/89	89/89	89/89	85/89	85/89	85/89	85/89
UNH 208*		103/103	86/100	103/103	103/103	103/103	103/103	103/103	103/103	100/103	86/103	86/103	86/103
UNH 211		126/126	117/131	126/126	126/126	126/126	126/126	126/126	126/126	126/131	117/126	126/131	126/131
UNH 228		228/228	224/228	228/228	228/228	228/228	228/228	228/228	228/228	224/228	224/228	224/228	224/228
UNH 189*	B (334 X 527)	153/153	158/168	153/153	153/153	153/153	153/153	153/153	153/153	153/158	153/158	153/158	153/158
UNH 203*		85/85	85/101	85/85	85/85	85/85	85/85	85/85	85/85	85/85	85/85	85/100	85/100
UNH 208*		86/86	86/100	86/86	86/86	86/86	86/86	86/86	86/86	86/86	86/100	86/100	86/100
UNH 211		129/129	117/131	129/129	129/129	129/129	129/129	129/129	129/129	117/129	117/129	117/129	117/129
UNH 228		228/228	224/228	228/228	228/228	228/228	228/228	228/228	228/228	228/228	224/228	224/228	224/228
UNH 189*	C (094 X 817)	153/153	153/178	153/153	153/153	153/153	153/153	153/153	153/153	153/153	153/153	153/153	153/153
UNH 208*		84/84	93/103	84/84	84/85	84/86	84/87	84/88	84/89	84/93	84/93	84/93	84/103
UNH 228		228/228	214/224	228/228	228/228	228/228	228/228	228/228	228/228	224/228	224/228	224/228	214/228
UNH 189*	D. (A861 X 817)	168/168	153/178	168/168	168/168	168/168	168/168	168/168	168/168	168/178	153/168	153/168	153/168
UNH 208*		100/100	93/103	100/100	100/100	100/100	100/100	100/100	100/100	93/100	93/100	93/100	100/103
UNH 228		214/214	214/224	214/214	214/214	214/214	214/214	214/214	214/214	214/224	214/224	214/224	214/224
UNH 189*	E (BE59 X 817	168/168	153/178	168/168	168/168	168/168	168/168	168/168	168/168	153/168	168/178	153/168	153/168
UNH 203*		83/83	83/89	83/83	83/83	83/83	83/83	83/83	83/83	83/89	83/83	83/89	83/83
UNH 208*		111/111	93/103	111/111	111/111	111/111	111/111	111/111	111/111	93/111	103/111	103/111	103/111
UNH 211		136/136	126/136	136/136	136/136	136/136	136/136	136/136	136/136	126/136	136/136	126/136	126/136
UNH 228		214/214	214/224	214/214	214/214	214/214	214/214	214/214	214/214	214/214	214/224	214/224	214/224

The touchdown PCR programme designed to amplify products in a multiplex PCR was successful. However, only triplex and duplex PCR reactions were successful in parentage analysis of clonal lines A, B, E and C, D respectively (Table 2.8). Although on some occasions artifacts were observed, these were easily resolved by comparison with the results of single locus PCR.

In all clonal lines, all clonal individuals inherited maternal alleles, however control animals inherited one of the alleles from the founder female and either one from the donor male. Although the sample size was small, the inheritance among the control individuals in most cases was more or less equal at both alleles (Table 2.8).

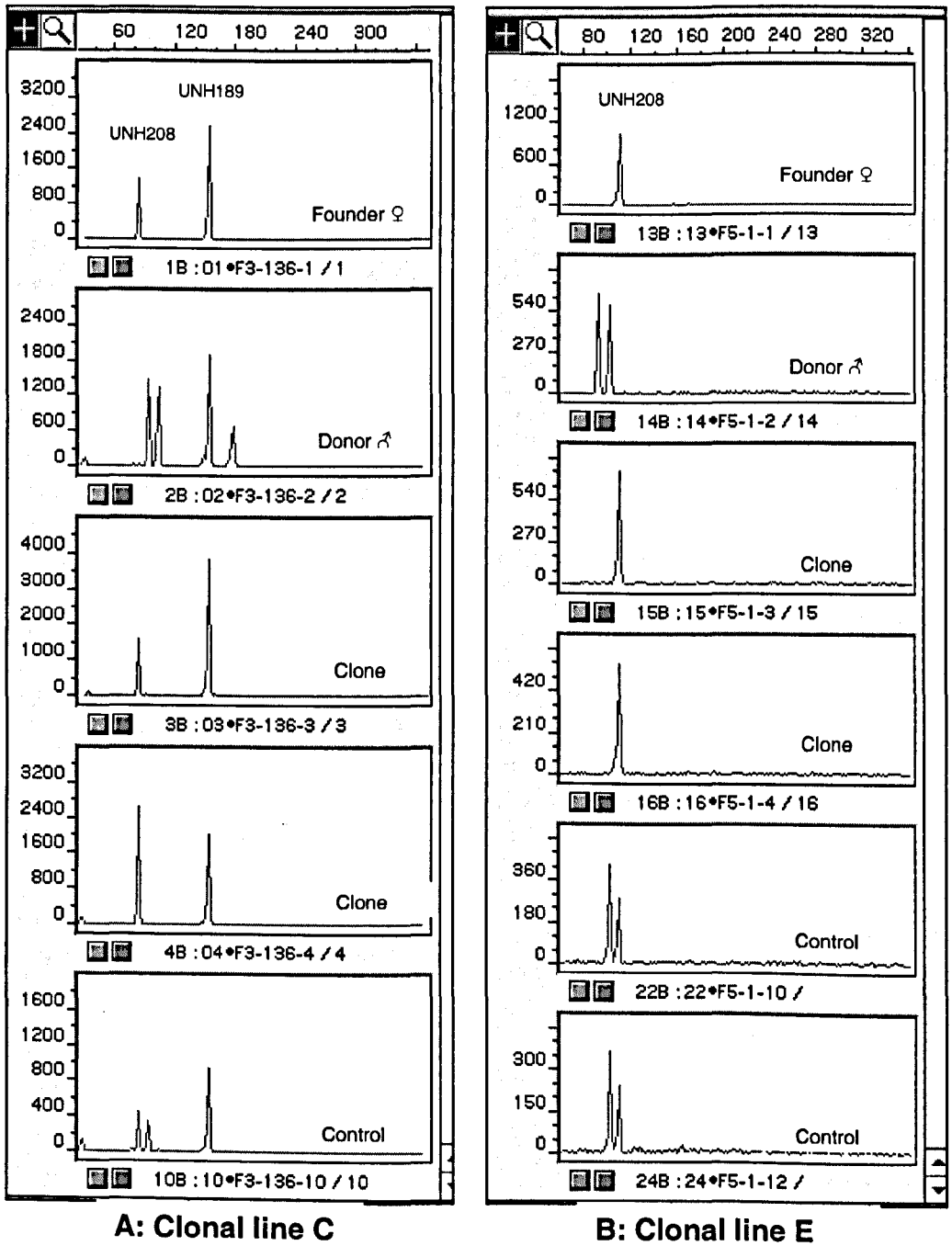


Fig. 2.6. Electropherograms generated by GeneScan™ (V. 2.1) software showing an example of a duplex PCR amplification at UNH189 and UNH208 loci in clonal line C and a single locus PCR amplification at locus UNH208 in clonal line E sample set of *Oreochromis niloticus*. A: family set of clonal line C; B: family set of clonal line E.

2.4. Discussion

In this study, the main aim was production and propagation of clonal lines of Nile tilapia which were to be used for different immunological assays, therefore, the main emphasis was given to propagation of clonal lines rather than standardisation of the technique. Gynogenesis is now a routine chromosome set manipulation technique and has been applied to a large number of different fish species with variable degrees of success.

Gynogenesis was carried out according to the procedure described by Sarder *et al.* (1999). The UV irradiation of sperm for 2.0 minutes at 250-265 $\mu W\ cm^{-2}$ successfully irradiated the sperm genome as shown by the lack of survival in the UV control group. A higher UV dose of 300-310 $\mu W\ cm^{-2}$ has also been used for Nile tilapia sperm irradiation (Hussain *et al.*, 1993). The effect of UV intensity and duration were not optimised in this study, therefore its implication on the survival rate cannot be discussed. Sarder *et al.* (1999) observed a higher survival rate in mitotic gynogenetics when a lower UV dose was used and suggested that higher UV dose caused more damage to sperm, resulting in poor fertilisation rate and consequently in poor survival rates in mitotic gynogenetics.

In the present study, clonal lines A, B and C were second generation clones produced from clonal females from the respective clonal lines by meiotic gynogenesis followed by sibmating. Clonal lines D and E were first generation clones produced from XX mitotic daughters of XY neofemales. A low survival rate was observed in both pigmentation and YSA stages in the meiotic gynogenetics but also in some of the respective control groups. Poor survival in meiotic gynogenetics as well as in the control group might be

due to poor egg quality of the females used as well as low resistance to heat shock. A significant difference in survival rate in the different stages of embryonic development was observed only in clonal line A. The lack of significant differences probably reflects a high variation between egg batches. The rate of survival at both pigmentation and YSA was not significantly different among the five clonal lines.

Comparison of survival data between first generation (Sarder, 1998) and second generation (this study) of clonal lines A, B and C at pigmentation and YSA showed that clonal line A had similar survival in both generations while in clonal line B and C, survival has dropped from first to second generation in both the stages. Significantly higher survival was observed at both developmental stages when sibmating was used to produce clonal lines than when meiotic gynogenesis was done. Therefore, sibmating seems to be a better option to propagate clonal lines than repeated meiotic gynogenesis.

The sex ratios of inbred and outbred clonal lines were 100% females. In Nile tilapia, both mitotic and meiotic gynogenetics should be exclusively female (Penman *et al.*, 1987a) as males in this species are heterogametic, XY and females are homogametic, XX (Jalabert *et al.*, 1974; Hopkins, 1979). The presence of males among mitotic gynogenetics has been observed in Nile tilapia (Mair, 1988) and in common carp (Komen and Richter, 1993). Sarder *et al.* (1998) reported presence of 25% males in one *O. niloticus* clonal line produced by meiotic gynogenesis from a mitogynogenetic female. The genetic sex determination model often fail to explain the occurrence of males in gynogenetic clonal line. Autosomal (Hussain *et al.*, 1994a) and environmental specially temperature (Baroiller *et al.*, 1995a,b; Abucay *et al.*, 1999) effects on sex

determination in this species have been reported. These hypotheses explain the presence of males in gynogenetic clonal lines of Nile tilapia.

Most earlier experiments concerning inheritance in genetically manipulated fish have involved the application of multilocus DNA fingerprinting with various minisatellite probes, randomly amplified polymorphic DNA or analysis with enzymatic markers (e.g. Carter *et al.*, 1991; Hussain *et al.*, 1993; Sarder *et al.*, 1999; Volckaert *et al.*, 1994; Van Eenennaam *et al.*, 1996). Parentage assignment can be achieved with any type of genetic marker provided that it is sufficiently polymorphic and for that reason microsatellites have been preferred recently to isozymes (Gerber *et al.*, 2000). Although several studies have been conducted using polymorphic microsatellites for paternity analysis in both plants (Dow and Ashley, 1998; Streiff *et al.*, 1999) and animals (Moran and Garcia-Vazquez, 1998; O'Reilly *et al.*, 1998), few studies have analyzed parentage in genetically manipulated populations. Galbusera *et al.* (2000) and Peruzzi and Chatain (2000) successfully used polymorphic microsatellite loci to verify maternal inheritance in gynogenetic populations of African catfish and European sea bass respectively.

In the present study, based on the single locus optimisation, duplex and triplex PCR using microsatellites was successful to verify clonal status. No paternal transmission or heterozygosity were observed in any of the clonal individuals tested. However, paternal transmission and heterozygosity of microsatellite loci among putative mitotic gynogenetic progeny were observed in African catfish (Galbusera *et al.*, 2000) and European seabass (Peruzzi and Chatain, 2000).

Multiplexing PCR is often difficult, requiring specific primer design and optimisation of conditions (Neff *et al.*, 2000). Since its initial description (Chamberlain *et al.*, 1988)

PCR multiplexing has been applied in a wide variety of applications in many different organisms, including humans, to study mutations (Shuber *et al*, 1993), deletion analysis (Henegariu *et al.* 1994), polymorphisms (Mutirangura *et al.*, 1993; Rithidech *et al.*, 1997), population genetics and parentage (Herbinger *et al.*, 1995; Wenburg *et al.*, 1996; Fishback *et al.*, 1999; Leneuve *et al.*, 2001); Shikano and Taniguchi, 2002) and pathology (Alegre *et al.*, 2001; Buerger *et al.* 2001). In the present experiment, pre-designed primers which were not designed specifically to work in multiplex were used in multiplex PCR. Therefore in some occasions, multiplex PCR was not successful. In some cases the same sized alleles were produced by different loci. In such cases where there was ambiguity between the two loci having same size alleles, the PCR and analysis was performed separately.

The overall versatility of microsatellites clearly indicates their general superiority over other forms of genetic markers. Compared to allozymes, microsatellites are a non-lethal technique, allowing selection and preservation of parental types and the maintenance of progeny for further investigation. When compared to the Southern hybridisation-based multilocus fingerprinting techniques, PCR-based microsatellites are more sensitive, require less DNA and are quicker. For each cross, microsatellites do not require identification of parent-specific bands as is the case when using RAPD markers, and therefore, direct identification of parent-specific alleles is possible without prior progeny testing (Ferguson *et al.*, 1995; Peruzzi and Chatain, 2000). Microsatellites also have high reproducibility (Lynch and Milligan, 1994) and can also distinguish between mitogynogenetics and meiogynogenetics (Ferguson *et al.*, 1995; Ezaz, 2002).

The application of polymorphic microsatellite loci for the parentage analysis of genetically manipulated population of *O. niloticus* was able to verify the success of this technique. Once optimised, the approach of amplification of several microsatellite loci in multiplex PCR was also found to be beneficial considering the time, efforts and costs.

From the above discussion, it can be stated that production and propagation of fully inbred clonal lines by combined techniques of gynogenesis and sex-reversal was successful and their verification using several microsatellite loci were also successful. Using cross breeding between two clonal lines an outbred clonal line was also established. Therefore, these fully inbred and outbred clonal lines were suitable for subsequent research involving specific immune responses and disease resistance in this species.

CHAPTER 3

SPECIFIC HUMORAL IMMUNE RESPONSE OF CLONAL LINES IN NILE TILAPIA *Oreochromis niloticus* TO SRBC, DNP-KLH AND TNP-LPS

3.1. Introduction

The transition of fish from a natural environment to aquaculture is accompanied by a number of changes, one of which is their increased susceptibility to disease due to increased stocking densities, frequent degradation of environmental quality, mixing of populations of different origin and manipulations (Chevassus and Dorson, 1990). The increase in fish culture over the last several decades has led to an increase in the variety and incidence of disease (Kinghorn, 1983; Shoemaker *et al.*, 2001). Fish may suffer from diseases not usually found in the wild population (Herman, 1970). Under crowded culture conditions, as a result of intensification of fish culture, seriousness of diseases often leads to epidemics and may cause severe losses (Meyer and Hoffman, 1976). It has been estimated that about 10% of all cultured fish die because of infectious diseases (Plumb, 2001).

Control of disease is of considerable importance in fish farming. The need to control disease has led to the development of a number of possible interventions i.e. specific diagnosis methods, sanitary prophylaxis and disinfections, and chemotherapy, particularly antibiotics and vaccination (Price, 1985).

A number of constraints have made some of these interventions difficult and expensive. Prophylaxis is based on a strict sanitary isolation, which is relatively easy in terrestrial species but is often more difficult in the aquatic environments. In aquatic environments, other fish species or even other invertebrates and vertebrates constitute potential contaminating agents which are difficult to eliminate. Traditional methods of control by chemotherapy using antibiotics are mostly applied orally and are not always effective, may cause pollution, can be costly and may be subject to prohibitive

legislation. In addition, antibiotic therapy may not be effective against antibiotic resistant bacteria (Chevassus and Dorson, 1990). Despite impressive technical progress, commercially available vaccines are mostly against bacterial diseases (Adams *et al.*, 1997; Ellis, 1997). One of the problems with viral vaccine production is the relatively high cost of traditional virus production in tissue culture system (Van Muiswinkel *et al.*, 1999).

An alternative approach for disease control has been recommended by the Food and Agriculture Organisation (FAO, 1972), which is to develop disease resistant strains through the genetic manipulation of fish stocks. This is why geneticists and pathologists are interested in the search for intrinsic resistance factors responsible for protecting individual fish during part or the whole of their biological cycle in an aquaculture environment (Chevassus and Dorson, 1990). Breeding for disease resistance can supplement the improvement of management methods to control infectious disease in aquaculture (Eide *et al.*, 1994).

To develop such strains of fish it is necessary first, to investigate and describe the amount and type of genetic variation in susceptibility to disease and second, to consider how to utilise any variation in resistance found. The genetic basis of susceptibility and resistance to disease in fish, including inter-specific variation, variation between populations and variation among populations have been extensively reviewed by Price (1985) and Chevassus and Dorson (1990).

In certain selection programmes to increase disease resistance in fish, the level of resistance to pathogens has been increased but the resistance is never absolute. One reason for this is the multitude of factors likely to be involved in determining whether a

fish is resistant or not. Resistance is often measured by mortality over a given period of time following exposure to infection. Such an approach is an unfortunate necessity, since there can be no other way to access resistance in fish (Price, 1985). There is insufficient knowledge of the epidemiology of some diseases to enable a controlled experimental infection. It may also be impossible to exclude death from other causes (Kirpichnikov *et al.*, 1979). Thus, the resistance being measured is often several steps removed from the process of infection. A better approach to selection would be indirect select for some specific physiological characters or genetic markers, if these could be identified. (Purdom, 1974). Markers can be simple phenotypic features such as colour or scale pattern, more complex polymorphic microsatellite loci or characters of functional importance such as immune parameters (Wiegertjes *et al.*, 1996a). Wolf (1953) listed some of those factors, all of which probably have a genetic basis, such as number and activity of leucocytes, ability of macrophages to digest phagocytosed bacteria, bactericidal properties of blood serum, ability to form antibody, and many others.

Indirect selection criteria for disease resistance, such as those based on immune response parameters, can lead to individual identification of potentially healthy fish. They have the added advantage that difficult experimental infection models, necessary for direct selection schemes, are not needed (Chevassus and Dorson, 1990).

Lysozyme activity (Roed *et al.*, 1993 a,b; Lund *et al.*, 1995b) and the complement system (Jensen and Koch, 1991; Roed *et al.*, 1990, 1992, 1993b; Slierendrecht *et al.*, 1993, 1996; Wiegertjes *et al.*, 1993b; Hollebecq *et al.*, 1995) are the two most widely used non-specific defence mechanism characteristics that have been linked to

differences in disease resistance between fish strains.

Antibody response has also been considered to be a marker trait for disease resistance in farmed animals including fish (commented by Eide *et al.*, 1994). Using specific antibody production to natural pathogens as genetic markers has the difficulty of distinguishing between antibody production due to immunisation and the infection caused by naturally occurring pathogens. If test antigens are used, the interference with natural pathogens can be avoided and antibody response can be regarded as the result of immunisation. Another advantage of using test antigens is that they are immunologically well characterised (Eide *et al.*, 1994).

A large number of studies have focused on modification of antibody mediated immunity by selective breeding. The best known examples are selection in mice for high and low antibody production to sheep red blood cells (SRBC) initiated by Biozzi *et al.* (1979). Fifteen generations of divergent selections resulted in lines assumed to be homozygous for genes determining the magnitude of antibody response. The need for uniformity has been the driving force behind the development of inbred lines of mice. Van der Zijpp and Nieuwland (1986) also initiated a divergent selection for anti-SRBC response in chicken. Using the information from nine generations a substantial genetic influence on high and low immune responsiveness was calculated (Pinard *et al.*, 1992).

Despite some conflicting results, examining possible associations between inherited resistance to infectious disease and immune parameters (Gjedrem *et al*, 1991; Balfry *et al.*, 1994), marker-assisted selection based on immunogenetic markers remains an attractive approach to the genetics of disease resistance in fish (Wiegertjes *et al.*, 1996a).

However, although significant genetic variation has been found in immune responses,

more knowledge is needed on the genetic correlation of immune parameters with disease survival before these correlation can be applied for indirect selection (Fjalestad *et al.*, 1993). Preliminary investigations in standardised laboratory fish lines would allow the much needed thorough analysis of the relationship between immune parameters and disease resistance (Wiegertjes *et al.*, 1996a). Despite the fact that inbred lines of mice have led to rapid progress and have become indispensable in every branch of biomedical science (Klein, 1986), the use of inbred fish lines in immunological research is recent (Wiegertjes *et al.*, 1995a; Sarder *et al.*, 2001). Such inbred lines can have a clear experimental value in unravelling environmental and genetic effects and their stable genetic constitution can be invaluable for a thorough immunological analysis of disease resistance. They will allow comparison of data between subsequent generations (Wiegertjes *et al,* 1994). Homozygous fish lines, especially congenic lines, are expected to be powerful tools for the identification of single gene with major effects on resistance to infectious diseases (Wiegertjes *et al.*, 1996a,b). Inbred laboratory fish lines, selected for high or low immune responsiveness and in which these phenotypes have clearly been defined, can offer appropriate models for investigating the genetic factors of resistance to infections, similar to the role played by comparable mouse lines (Mouton *et al.*, 1988).

Streisinger *et al.* (1981) reported the use of artificial gynogenesis for the rapid production of fully inbred fish lines in just two generations in zebrafish. Its application resulted in fully inbred lines, each consisting of genetically identical animals. They enable the production of an almost unlimited number of fish expressing the same unique genotype (Wiegertjes *et al*, 1994). Similar reproduction methods have now been

developed for a number of fish species including commercially important species such as common carp (Nagy *et al*, 1978; Komen *et al.*, 1988, 1991) and Nile tilapia (Hussain *et al*, 1998; Sarder, 1998).

Test antigens have been used in several studies concerning antibody response in inbred mouse lines (Biozzi *et al.*, 1979) and in inbred chicken lines (Van der Zijpp and Nieuwland, 1986). Induced gynogenesis was used in order to obtain genetically standardised inbred common carp lines and these were divergently selected for high and low immune responsiveness lines to T-dependent hapten-carrier conjugate, dinitrophenyl-keyhole limpet haemocyanin (DNP-KLH) for the study of immunogenetic regulation of disease resistance (Wiegertjes *et al.*, 1994, 1995a,b,c, 1996b).

3.1.1. The aim of the study

The aim of the experiment was to study and compare the specific immune responses of the different experimental groups of Nile tilapia using a variety of different test antigens as tools. Two inbred clonal lines of Nile tilapia, an outbred clonal line (produced by crossing female and male from two inbred clonal lines) and an unrelated control group consisting of normal outbred Nile tilapia were used. The specific immune responses of those different experimental fish groups were measured using sheep red blood cells (SRBC), hapten-carrier conjugates thymus-dependent (TD) DNP-KLH and thymus-independent (TI) trinitrophenyl lipopolysaccharide (TNP-LPS) antigens. SRBC is a polyvalent multideterminant antigen. Studies on mice (Biozzi *et al.*, 1979) and chickens (Van der Zijpp and Nieuwland, 1986) facilitated the selection of SRBC as an antigen in this study. Haptens are chemically defined substances of low molecular weight and cannot induce a specific immune response by itself. Haptens can induce

specific immune response only when it is coupled to a carrier protein molecule. DNP-KLH requires direct contact with T-helper cells, not simply exposure to T-helper cell derived cytokines. TNP-LPS can activate B lymphocytes to produce antibodies without the help of T-helper cells (Kuby, 1997). Possible correlation between simple hapten determinants and immune response (Ir) genes of major histocompatibility complex (MHC) Class II and the study of Wiegertjes *et al.* (1994, 1995a,b,c) in common carp prompted the choice of hapten-carrier complexes (DNP-KLH and TNP-LPS) as antigens.

3.2. Materials and methods

3.2.1. Stock of experimental fish and their maintenance

The Nile tilapia, used in the experiments came from the Tilapia Reference Collection at the Institute of Aquaculture, University of Stirling. Their production, propagation and maintenance were described in Chapter 2 of this thesis.

Two inbred clonal lines of Nile tilapia, an outbred clonal line produced by crossing between a male and a female from the two inbred clonal lines and an unrelated control group of fish comprising of normal Nile tilapia were used. The two clonal lines are identified by the tag numbers of their mitotic founder mothers. Thus the two clonal lines are 009-356-316 and 010-036-092 and will be referred to as clonal line A and clonal line B respectively for easy description. The outbred clonal line will be referred to as OBC (AXB) and the unrelated normal Nile tilapia as unrelated control URC.

Fish from all experimental groups were of 4-5months of age and the average weight was 65 g. The fish were kept in the same recirculating system at the tropical aquarium facility of the Institute of Aquaculture during the course of the experiment.

All procedures of fish anaesthesia and immunisation were performed under project and personal licences issued by the U.K. Home Office.

3.2.2. Immunisation of fish with sheep red blood cells (SRBC)

Seventy-eight fish from each experimental group were used. All fish were split into six tanks with 13 fish from each experimental group in the same tank. Fish from three tanks were immunised with SRBC in adjuvant and fish from the other three tanks were injected with phosphate buffered saline (PBS; 0.02M, pH 7.2) in adjuvant and were

treated as non-immunised control. Therefore, in total, 39 fish from each group were immunised with SRBC and 39 fish were kept as non-immunised controls.

Sheep blood was purchased as 50% (v/v) solution in Alsever solution (Diagnostic Scotland, Lanark, Scotland). SRBC was washed three times with PBS at 250 g for 7 min and finally resuspended in PBS as 20% (v/v) solution as suggested by Sailendri and Muthukkaruppan (1975a). This was mixed with the adjuvant "Montanide" (Aquatic Vaccines Ltd., UK) at a ratio of 3:7 and mixed well by vortexing to form a stable emulsion. An i.p. injection of 0.2 ml of the emulsion was given to immunise the fish. Fish injected with 0.2ml of PBS in adjuvant were considered as non-immunised.

On the fourth day after immunisation and thereafter at 7 day intervals, blood samples were collected from different experimental fish groups. Those fish were killed by immersion in concentrated benzocaine solution. Peripheral blood and spleen and head kidney tissues were used for lymphocyte isolation and the isolated lymphocytes were used in a plaque forming cell (PFC) assay to determine the numbers of antibody secreting cells (ASC). This schedule of sampling was continued until the experiment was terminated.

Blood samples were collected at 7 day intervals post-immunisation by caudal venipuncture and collected blood was kept at 4°C overnight. The tubes were centrifuged at 9,300 g for 5 min to separate serum. Collected serum samples were stored at -70°C until analysed for antibody titre using an enzyme linked immunosorbent assay (ELISA).

Antibody secreting cells are first formed in lymphoid tissues and then release the antibodies in blood which subsequently increase the serum antibody titre. Sailendri and Muthukkaruppan (1975a) reported that in Mozambique tilapia, the maximum numbers

of PFC were observed three days prior to the peak serum antibody levels. Therefore, the number of antibody secreting cells were determined three days prior to measuring the serum antibody titre in all experiments.

3.2.2.1. Lymphocyte isolation

Collection of spleen and head kidney tissues from fish and isolation of lymphocytes were performed under a laminar flow hood (Gelaire, ICN Flow) to avoid contamination.

Lymphocytes were separated from blood, spleen and head kidney using the method described by Findlay (1994). This method was originally described by Boyum (1968) for the isolation of mammalian mononuclear cells from circulating blood and bone marrow.

Blood was removed from the caudal vein and diluted 1:4 in L15 Leibovitz medium (Sigma) containing 20 iu heparin (Sigma). Head kidney and spleen tissue were gently pressed through a nylon mesh into 5 ml of L15 Leibovitz medium containing heparin, to make a cell suspension.

The diluted tissue cell suspension was then carefully layered over 7 ml of lymphocyte separation medium, Histopaque (Sigma), in universal tubes and centrifuged at 1100 g for 45 min at 4°C. The buffy coat layer at the L15 Leibovitz-Histopaque interface was carefully removed using a sterile Pasteur pipette. Cells were washed once in 10 ml L15 Leibovitz and resuspended in 1 ml L15 Leibovitz containing 5% heat inactivated foetal bovine serum (FBS, Sigma). The number of cells were counted, the concentration adjusted according to the assay, and resuspended in L15 Leibovitz containing 5% heat inactivated FBS, and supplemented with penicillin/streptomycin (100 units of penicillin and 50 µg of streptomycin per ml of medium; Sigma), L-glutamine (2mM; Sigma) and

sodium pyruvate (1% v/v; Sigma) for experimental use.

3.2.2.2. Optimisation of lymphocyte concentration and serum supplement for PFC assay

The cell and serum supplements used in the PFC assay were optimised in a trial experiment. Lymphocytes from spleen and head kidney were used. Four different lymphocyte concentrations ($5x10^5$ ml^{-1}, $1x10^6$ ml^{-1}, $2.5x10^6$ ml^{-1} and $5x10^6$ ml^{-1}) and four different serum supplements (2%, 5%, 10% and 20%) were used. The result of this optimisation trial is described in Section 3.3.1.1.

3.2.2.3. Plaque forming cell (PFC) assay

This assay was done on the fourth day after immunisation and thereafter at 7 day intervals during the course of the experiment. i.e. on days 4, 11, 18 and 25 post-immunisation.

The microwell haemolytic PFC assays of Kappler (1974) and Findlay (1994) were followed for PFC assay using lymphocytes from peripheral blood, spleen and head kidney of Nile tilapia immunised with SRBC.

Concentrations of lymphocyte cell suspensions from blood spleen and head kidney were adjusted to $2.5x10^6$ cells ml^{-1} (optimised in trial experiment as shown in Section 3.3.1.1.) in L-15 Leibovitz medium and 200 µl of this cell suspension was added per well (three wells per tissue) to the wells of a 96 well flat bottom cell culture plate (Nunc ™, Denmark) giving final *in vitro* concentrations of $5x10^5$ cells per well. The plates were incubated overnight in a moist box at 27°C and the PFC assay was performed on the next day.

SRBC (Diagnostic Scotland) was washed twice in PBS and re-suspended to 20%. A mixture of 20% SRBC and 10% complement solution was prepared. The complement was obtained by collecting serum from non-immunised Nile tilapia which was absorbed against SRBC before use (described later in this Section). The cell culture plates, after overnight incubation with the lymphocytes, were centrifuged at 250 g for 10 min at 4°C at an angle so that the cells formed pellets on the side of the wells. The plates were then inverted and the cell culture medium expelled with a brisk shake. Most of the residual medium was removed by blotting on paper towels. The plates were then gently shaken by vortexing to re-distribute the lymphocytes. The SRBC-complement mixture was added at 100 µl per well.

The plates were again shaken very gently on a vortexer to mix the contents of the wells. The plates were incubated undisturbed at 4°C for one hour. During this time, the cells settled as a monolayer on the bottom of each well. The plates were then warmed slowly by first standing at 21°C for 15 min and then incubated at 27°C for five hours in a moist box for the plaques to develop. The plaques were examined using an inverted microscope. The plaques appeared as clear spots in the red blood cell background and were counted under a dissecting microscope at x10 magnification.

The complement used in this assay was absorbed against SRBC to remove naturally occurring antibodies in Nile tilapia serum against SRBC which would otherwise cause a general lysis (Sailendri and Muthukkaruppan, 1975a). The complement used in the assay consists of serum obtained from non-immunised Nile tilapia collected over a period of weeks and stored at -70°C. A volume of SRBC, equal to 1/3-1/2 of the serum to be absorbed, was removed directly from the supplier's bottle and washed twice is PBS. The

serum was poured over the packed SRBC and mixed gently and incubated on ice for 30 min with periodic mixing to ensure the cells remained in suspension. The cell suspension was transferred into centrifuge tubes and centrifuged at 400 g for 10 min at 4°C. The serum was then separated and the centrifuged again to remove any remaining SRBC. The collected serum was stored at -70°C in 0.5 ml aliquots.

3.2.2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

An indirect ELISA was used to measure serum antibody titres in the immunised fish against SRBC, following the protocol described by Findlay (1994) and Chen *et al.* (1996). Serum samples collected from the different fish groups each week post-immunisation was screened. This was carried out at 7 days intervals, i.e. on days 7, 14, 21 and 28 post-immunisation.

Serum samples from the different groups of experimental fish were used in the same ELISA plate to remove potential bias from any differences between ELISA plate readings.

Ninety-six well Hydrophilic Immunol 4 HBX ELISA plates (Dynex Technologies Inc., Chantilly, USA) were pre-coated with 1% Poly-L-Lysine in coating buffer (0.125 M carbonate-bicarbonate buffer, pH 9.6) at 100 µl per well and incubated for 2 hours at 21°C. Plates were washed with low salt wash buffer [LSW: 0.02M Trizma base, 0.38M NaCl and 0.05% (v/v) Tween 20 pH 7.4]. The plates were coated with 100 µl per well of 0.5% (v/v) SRBC in PBS for 2 hours at 21°C. The plates were washed three times with LSW buffer. Non-specific binding sites were blocked by adding 250 µl per well of a 3% (w/v) casein solution (dried skim milk, Marvel, Cadburys, UK). The plates were

kept overnight at 4°C. Plates were washed three times with LSW buffer and a 100 µl sample of a two fold serial dilution of serum, diluted in PBS was added per well. Only PBS was added to the control wells. The plates were incubated for one hour at 21°C and then washed five times with high salt wash buffer [HSW: 0.02M Trizma base, 0.5M NaCl and 0.05% (v/v) Tween 20, pH 7.8] with a 5 min soak on the last wash. Undiluted mouse anti-tilapia monoclonal antibody (MAb) cell culture supernatant (a mixture of M3 against the light chain and M25 against the heavy chain cells of Nile tilapia IgM, Aquatic Diagnostics Ltd. Stirling, Scotland) was added at 100 µl per well and incubated for 1 hour at 21°C. The plates were washed five times with HSW buffer as before followed by adding 100 µl per well of anti-mouse IgG whole molecule monoclonal antibody conjugated to alkaline phosphatase (Sigma), diluted 1:1000 in PBS. The plates were incubated for 1 hour at 21°C and washed five times with HSW buffer as before. Then 100 µl of *p*-Nitrophenyl phosphate, disodium (Sigma) at 1.0 mg ml^{-1} in 0.05M diethanolamine buffer (Sigma) containing 0.5mM $MgCl_2$, pH 9.8, was added per well. The plates were incubated at 20 min at 21°C and the reaction was stopped by adding 50 µl of 3N NaOH per well. The optical density (OD) was then read at 405 nm using a Titerteck Multiskan ELISA plate reader. Results that were three times greater than the background value (negative control wells) were considered positive.

The titres were expressed as $\log_2$ of the reciprocal of the highest dilution at the end point (i.e. the last value to give a positive result).

3.2.3. Immunisation of experimental fish with T-dependent antigen, dinitrophenyl-keyhole limpet haemocyanin (DNP-KLH)

Ninety six fish from each experimental group were used. All fish were distributed into six tanks with 16 fish from each experimental group in the same tank. Fish from three tanks were immunised with T-dependent antigen, hapten-carrier conjugate, DNP-KLH (400 DNP molecule per molecule of protein; Calbiochem, La Jolla, USA) and fish from the other three tanks were injected with PBS in adjuvant and were treated as non-immunised controls. Therefore, in total, 48 fish from each group were immunised with DNP-KLH and 48 fish were kept as non-immunised controls.

Immunisation of fish with DNP-KLH was performed following procedure described by Wiegertjes *et al.* (1994) with minor modifications. DNP-KLH in PBS was mixed with the adjuvant "Montanide" (Aquatic Vaccines Ltd., UK) at a ratio of 3:7 (v/v) giving a final concentration of 100 μg DNP-KLH ml^{-1} of prepared antigen. This was mixed well by vortexing to form a homogeneous stable emulsion. Fish were immunised with an i.p. injection of 0.2 ml of the emulsion. Fish injected with 0.2 ml of PBS in adjuvant were considered as non-immunised controls.

3.2.3.1. Enzyme Linked Immunospot (ELISPOT) assay

A modification of the ELISPOT assay described by Koumans van-Diepen (1995) was used to quantify anti-DNP antibody secreting cells (ASC) after immunisation with T-dependent antigen, DNP-KLH. The schedule of carrying out this assay was as described in Section 3.2.2.3, only sampling for this assay was continued for a longer period of time. The ELISPOT assay was performed on days 4, 11, 18, 25, 32, 39, 46 and 53 post-immunisation.

Ninety six well multiscreen filtration plates (Multiscreen R-HA, sterile plate with 0.45 µm surfactant-free mixed cellulose ester membrane, Millipore) were coated with dinitrophenyl-bovine serum albumin (DNP-BSA, 500 µg ml^{-1}) in PBS at 100 µl per well and plates were incubated overnight at 4°C. The plates were washed and incubated for 15 min with PBS at 21°C. Any remaining reactive sites in the wells were blocked by adding 1% bovine serum albumin (BSA) (w/v) in PBS at 250 µl per well and incubating the plates for 1 hour at 21°C. Lymphocyte cell suspensions from blood, spleen and head kidney in L-15 Leibovitz medium were adjusted to $5x10^6$ ml^{-1} and were added at 100 µl per well (5×10^5 cells per well). Plates were incubated for 9 hours at 21°C. Care was taken not to disturb the cell suspension during incubation. Plates were washed three times in PBS and anti-tilapia monoclonal antibody (Mab) undiluted hybridoma supernatant (see Section 3.2.2.4) was added at 150 µl per well. Plates were incubated overnight at 4°C to detect bound DNP-specific antibodies. After three washes in PBS, alkaline phosphatase conjugated goat anti-mouse whole molecule IgG (Sigma), diluted 1:1000 in tris buffered saline (TBS) (0.02 M Tris, 0.05 M NaCl, pH 7.5) was added at 100 µl per well and plates were incubated for one hour at 21°C. Plates were washed three times with TBS containing 0.005% (v/v) Tween-20 (TTBS) and spots were developed in substrate buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$ with 0.1% nitro blue tetrazolium [NBT] and 0.04% 5-bromo-4-chloro-3-indolyl-phosphate[BCIP] for 10 min at 21°C. The plates were thoroughly rinsed in double distilled water, dried at 21°C and spots were counted under low magnification. These spots appeared as deep purple spots.

3.2.3.2. Enzyme-Linked Immunosorbent Assay (ELISA)

The schedule of carrying out this assay was as described in Section 3.2.2.4, only sampling for this assay was continued for a longer period of time. An indirect ELISA was performed on days 7, 14, 21, 28, 35, 42, 49 and 56 post-immunisation.

The indirect ELISA was used to measure serum anti-DNP antibody titres following the procedure described by Wiegertjes *et al.* (1994) and Chen *et al.* (1996). All washes and incubations were the same as the ELISA protocol for measuring the antibody titre against SRBC described in Section 3.2.2.4 unless otherwise mentioned.

ELISA plates were coated with DNP-BSA; (containing >30 DNP molecules per molecule of protein Calbiochem, La Jolla, USA) at 100 µl per well (0.1 µg ml^{-1}) diluted in coating buffer (0.125 M carbonate- bicarbonate buffer, pH 9.6). Plates were incubated overnight at 4°C. Non-specific binding sites were blocked with 250 µl per well 3% (w/v) BSA (Sigma) in TTBS for 1 hour at 21°C. Serum samples were diluted in TBS and added to wells as describe earlier and only TBS was added to the negative control wells. This was followed by adding the mouse anti-tilapia MAb. Goat anti-mouse immunoglobulin G labelled with horseradish peroxidase (HRP anti mouse IgG; Diagnostic Scotland, Lanark, Scotland) diluted 1:1000 in TBS was added per well. The TMB (tetramethyl benzidine) dihydrochloride/citric acid chromogen-substrate buffer system was used to develop the assay. This was prepared by adding 150 µl chromogen (43 mM TMB dihydrochloride in 2M acetic acid) to 15 ml substrate buffer (0.1M citric acid, 0.1 M sodium acetate, pH 5.4 and containing 0.33% v/v H_2O_2. The plates were incubated for 10 min at 21°C. The reaction was stopped by adding 2M H_2SO_4 at 50 µl per well. Plates were read at 450 nm using an ELISA reader (Dynex technologies,

USA). Expression of results and endpoints were determined as described before.

3.2.4. Immunisation of experimental fish with T-independent antigen, trinitrophenyl- lipopolysaccharide (TNP-LPS)

Trinitrophenyl-lipopolysaccharide (TNP-LPS; *Escherichia coli* Serotype 0111:B4; 12 μg TNP/mg LPS; Sigma) was used as the T-independent antigen. Immunisation of fish, determination of anti-TNP serum antibody titre using an indirect ELISA and quantification of ASC by ELISPOT assay were the same as described for T-dependent antigen, DNP-KLH in Sections 3.2.3, 3.2.3.1 and 3.2.3.2 respectively.

3.2.5. Statistical analysis

The results of PFC, ASC and serum antibody titre of different experimental groups of fish were analysed by multiple comparison analysis (Bonferroni multiple analysis) using general linear model (Minitab V. 13).

3.3. Results

3.3.1. Immune response of Nile tilapia to Sheep Red Blood Cells (SRBC)

3.3.1.1. Optimisation of lymphocyte concentration and serum supplement for PFC assay

The results of the trial experiment to optimise the lymphocyte and serum supplement concentrations for the PFC assay are presented in Fig. 3.1. A lymphocyte concentration of $5x10^5$ ml^{-1} with all four serum supplements often failed to produce any PFC (data not shown) whereas, lymphocyte concentration of $5x10^6$ ml^{-1} with all four serum supplements produced too many overlapping plaques making it difficult to count accurately (also data not shown). When lymphocyte concentrations of $1x10^6$ ml^{-1} and $2.5x10^6$ ml^{-1} were used with four different serum supplements, the number of plaques increased significantly ($P<0.05$) with the increase in serum supplement over the 2%, 5% and 10% concentrations but differences were not significant when serum supplement was increased from 10% to 20%. Therefore, a lymphocyte concentration of $2.5x10^6$ ml^{-1} supplemented with 10% serum was chosen to be used in the main PFC assay (Fig. 3.1).

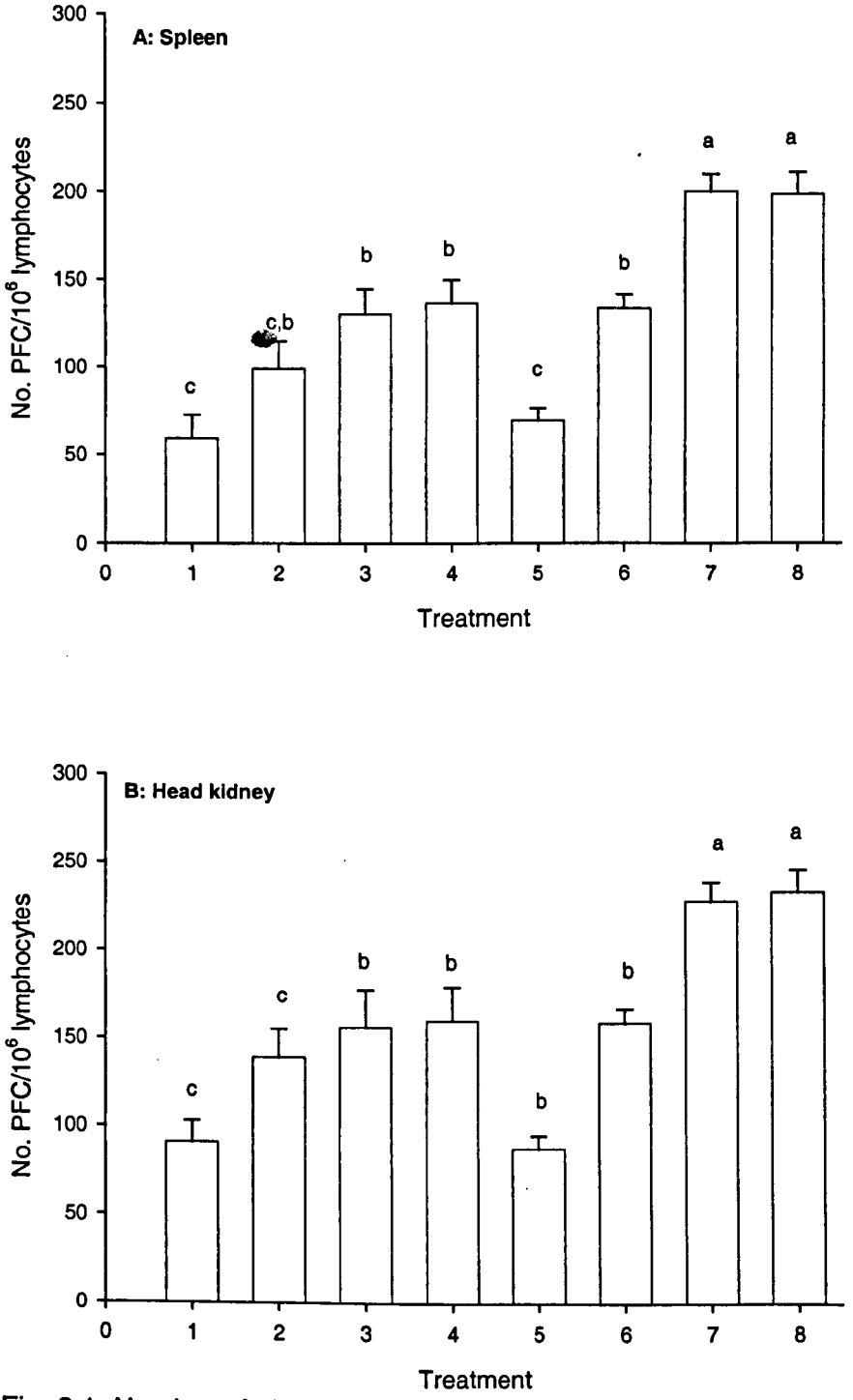


Fig. 3.1. Number of plaque forming cells (PFC) in Nile tilapia (*Oreochromis niloticus*) 10 days after immunisation with SRBC. Data presented as mean±SE PFC/10^6 lymphocytes; N=5. Different lower case letters indicate significant differences; A: Spleen; B: Head kidney. Treatments 1: 1x10^6 cells ml^{-1}+ 2% complement; 2: 1x10^6 cells ml^{-1}+ 5% complement; 3: 1x10^6 cells ml^{-1}+ 10% complement; 4: 1x10^6 cells ml^{-1}+ 20% complement; 5: 2.5x10^6 cells ml^{-1}+ 2% complement; 6: 2.5x10^6 cells ml^{-1}+ 5 % complement; 7: 2.5x10^6 cells ml^{-1}+ 10 % complement; 8: 2.5x10^6 cells ml^{-1}+ 20% complement.

3.3.1.2. Detection of anti-SRBC plaque forming cells (PFC)

Fish from all the experimental groups (i.e. clonal line A, clonal line B, OBC (AXB) and URC) elicited an immune response to the SRBC. The kinetics of the PFC response in the different organs in immunised fish of different groups of experimental fish is shown in Fig. 3.2.

Each group of immunised fish had a similar pattern of PFC response but which differed in magnitude. The number of PFCs response increased after immunisation and this level appeared to peak on day 11 post-immunisation in all groups of experimental fish. The number of PFCs then declined in all groups by day 25 post-immunisation day. The experiment was terminated at day 25 post-immunisation because on day 32, PFC response in clonal line B was very low and some individuals were non-responsive although clonal line A, OBC (AXB) and URC were still producing PFC in their spleen and their kidney.

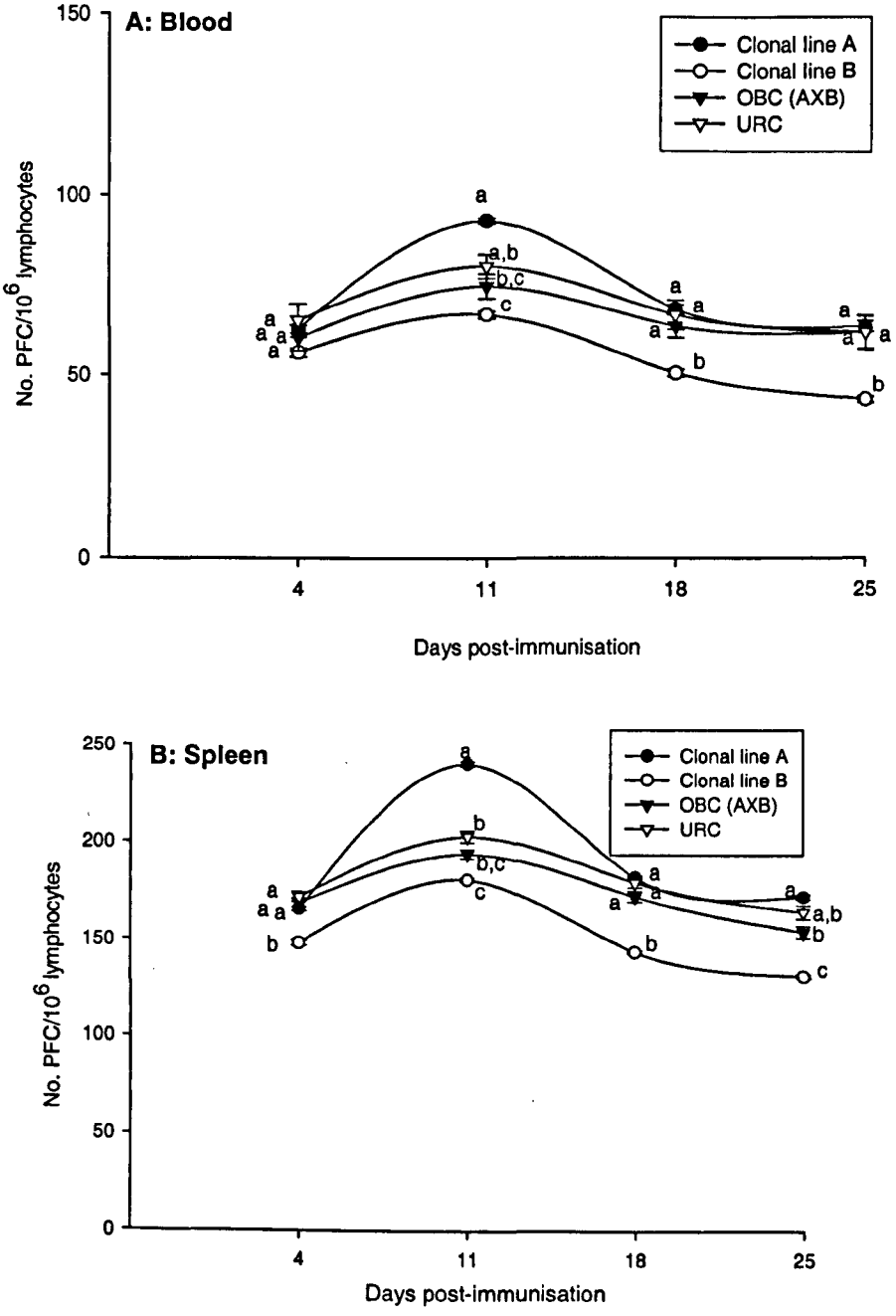


Fig. 3.2. The number of PFC in A: Blood; B: Spleen; C: Head kidney of different experimental groups of Nile tilapia (*Oreochromis niloticus*) (clonal line A, clonal line B, OBC (AXB) and URC) after immunisation with sheep red blood cells (SRBC). Data are presented as mean ± SE PFC/10^6 lymphocytes in triplicate wells (N=5), showing comparison among different groups at the same sampling day; different lower case letters indicate significant ($P<0.05$) difference.

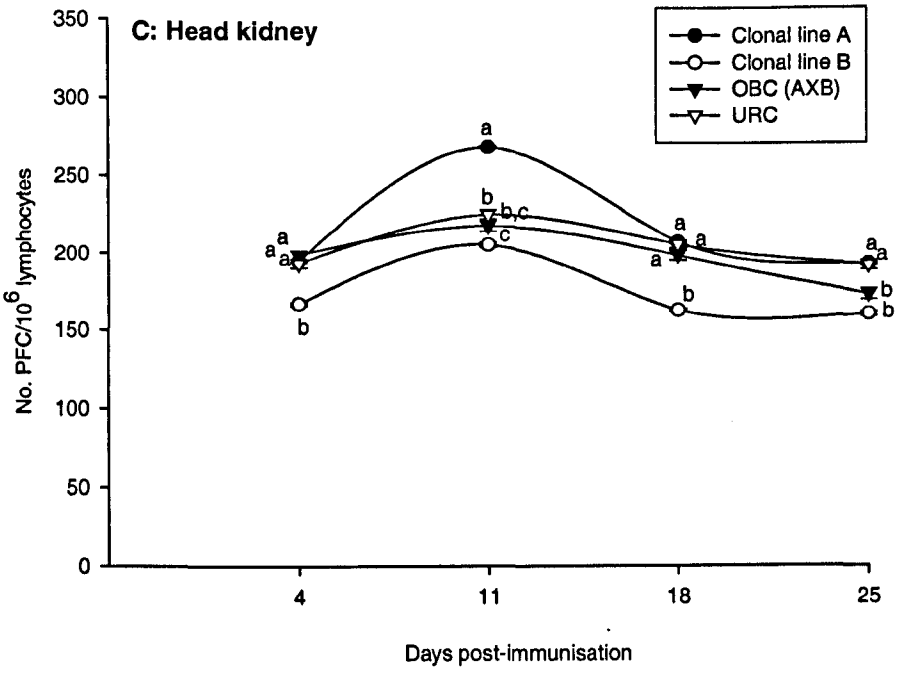


Fig. 3.2. The number of PFC in A: Blood; B: Spleen; C: Head kidney of different experimental groups of Nile tilapia (*Oreochromis niloticus*) (clonal line A, clonal line B, OBC (AXB) and URC) after immunisation with sheep red blood cells (SRBC). Data are presented as mean ± SE PFC/10^6 lymphocytes in triplicate wells (N=5), showing comparison among different groups at the same sampling day; different lower case letters indicate significant (P<0.05) difference.

The PFC response of clonal line A was significantly (P<0.05) higher than the response of clonal line B over the period of study, and this was true for all three tissues examined except the response in blood on day 4 post-immunisation. The response of clonal line A was significantly (P<0.05) higher than that of OBC (AXB) on day 11 in blood and on day 11 and on day 25 in the spleen and head kidney. Though the PFC response was also higher in clonal line A than the response of URC, the difference was not significant in blood at any times examined and was only significantly (P<0.05) different on day 11 of sampling in the spleen and kidney of these fish (Fig. 3.2).

The PFC response of clonal line B was lower at all times over the period of study compared to the response of OBC (AXB), but this difference was significant ($P<0.05$) on days 18 and 25 post-immunisation in blood, on days 4, 18, 25 post-immunisation in the spleen and on days 4 and 18 in the head kidney. Clonal line B had significantly ($P<0.05$) lower PFC than the response of URC on days 11, 18 and 25 post-immunisation in blood and at all times over the period of study in the spleen and the head kidney. Significant difference ($P<0.05$) was observed in the PFC response between OBC (AXB) and URC fish on day 25 post-immunisation in head kidney (Fig. 3.2).

The PFC responses observed in the blood, spleen and head kidney were significantly different from each other in all experimental groups of fish, with head kidney having the highest response followed by spleen then blood (Table 3.1).

Table 3.1. The number of PFC in experimental groups of Nile tilapia after immunisation with sheep red blood cells (SRBC). Data are presented as mean ± SE PFC/10^6 lymphocytes in triplicate wells (N=5). Different lower case letters indicate significant ($P<0.05$) differences among different tissues on same day; DPI: days post-immunisation.

Experimental Groups	DPI	Tissues		
		Blood [c]	Spleen [b]	Head kidney [a]
		Mean ± SE	Mean ± SE	Mean ± SE
Clonal line A	4	62.4 ± 1.0	165.6 ± 1.0	194.4 ± 1.0
	11	92.8 ± 0.8	240.0 ± 1.3	267.2 ± 0.8
	18	68.0 ± 1.3	181.6 ± 1.0	206.4 ± 1.0
	25	63.2 ± 1.5	172.0 ± 1.3	192.8 ± 0.8
Clonal line B	4	56.0 ± 1.3	148.0 ± 1.3	166.4 ± 1.0
	11	66.4 ± 1.0	180.8 ± 0.8	204.8 ± 1.5
	18	50.4 ± 1.0	143.2 ± 0.8	162.0 ± 0.9
	25	43.2 ± 0.8	130.8 ± 0.8	160.0 ± 1.3
OBC (AXB)	4	60.0 ± 3.6	168.8 ± 2.3	198.4 ± 2.7
	11	74.4 ± 3.4	193.6 ± 2.4	216.8 ± 3.4
	18	63.2 ± 3.2	172.0 ± 2.8	197.6 ± 3.2
	25	61.6 ± 4.7	153.6 ± 3.0	172.8 ± 3.4
URC	4	64.8 ± 4.6	172.0 ± 2.5	192.8 ± 2.7
	11	80.0 ± 3.3	202.4 ± 3.0	224.0 ± 2.8
	18	66.4 ± 4.1	179.2 ± 2.7	204.8 ± 2.3
	25	61.6 ± 4.5	164.0 ± 3.6	192.0 ± 2.8

No PFCs were detected in the tissues of non-immunised fish in any of the experimental groups of fish examined.

An example of plaques obtained by plaque forming cell (PFC) assay in clonal lines A and B is presented in Fig. 3.3.

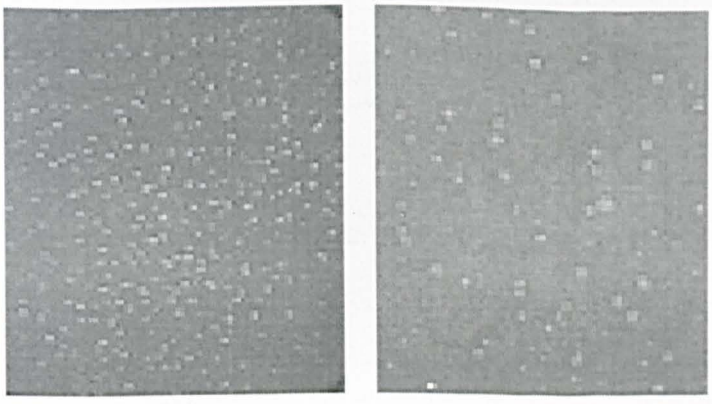


Fig 3.3. A typical well showing the plaques obtained in the plaque forming cell (PFC) assay from lymphocytes isolated from head kidney of A: clonal line A and B: clonal line B fish on 11 day post-immunisation with SRBC.

3.3.1.3. Measurement of anti-SRBC serum antibody titres in immunised fish

Serum antibody titres followed a similar pattern of kinetics in all experimental groups of fish over the course of the 28 days of study, however the magnitude of titre differed between the groups (Fig. 3.4). All the experimental groups of fish had the highest antibody titre on day 14 post-immunisation. After that, the titre declined in all groups of fish.

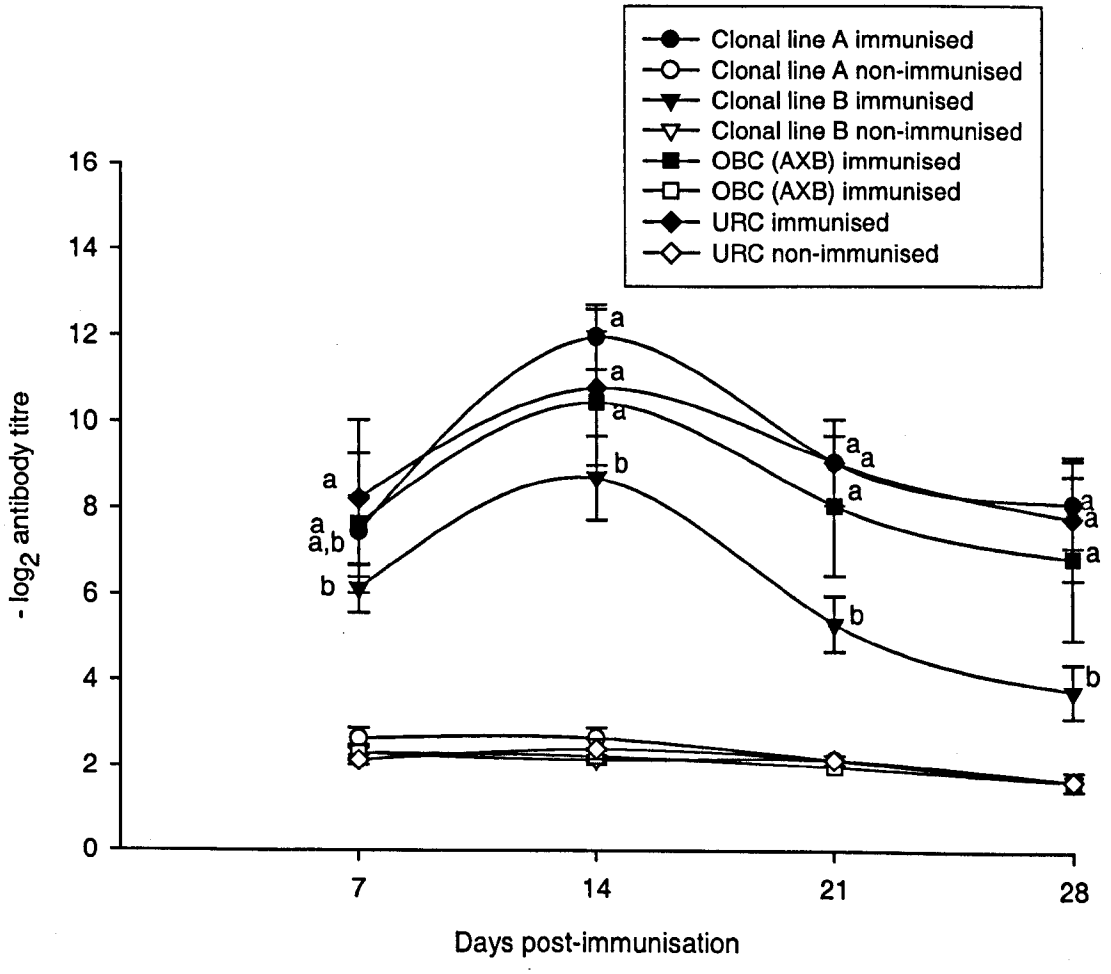


Fig. 3.4. Serum anti-SRBC antibody titres in different experimental groups of Nile tilapia (*Oreochromis niloticus*) following immunisation with sheep red blood cells (SRBC). Data are presented as mean ± SE of $-\log_2$ titre (N=12). Different lower case letters indicate significant ($P<0.05$) difference among different groups of fish on the same day post-immunisation.

The antibody titres of clonal line A was significantly ($P<0.05$) higher than the titres obtained for clonal line B over the period of the study. Clonal line A also had a higher

titres than OBC (AXB) fish at all times over the period of study and also had higher titres than URC fish onwards from day 7 post-immunisation, but these differences were not significant (Fig. 3.4).

Clonal line B had significantly ($P<0.05$) lower titres than both OBC (AXB) fish and URC fish over the period of study. The URC fish had slightly higher titres than OBC (AXB) fish but this was not significantly different (Fig. 3.4).

The non-immunised fish of clonal lines A and B, OBC (AXB) and URC had very low antibody titre against SRBC due to a non-specific response, but no difference was observed in this titre between the different groups of fish (Fig.3.4). Mean serum anti-SRBC titres of the experimental groups of fish are also presented in Appendix 1.

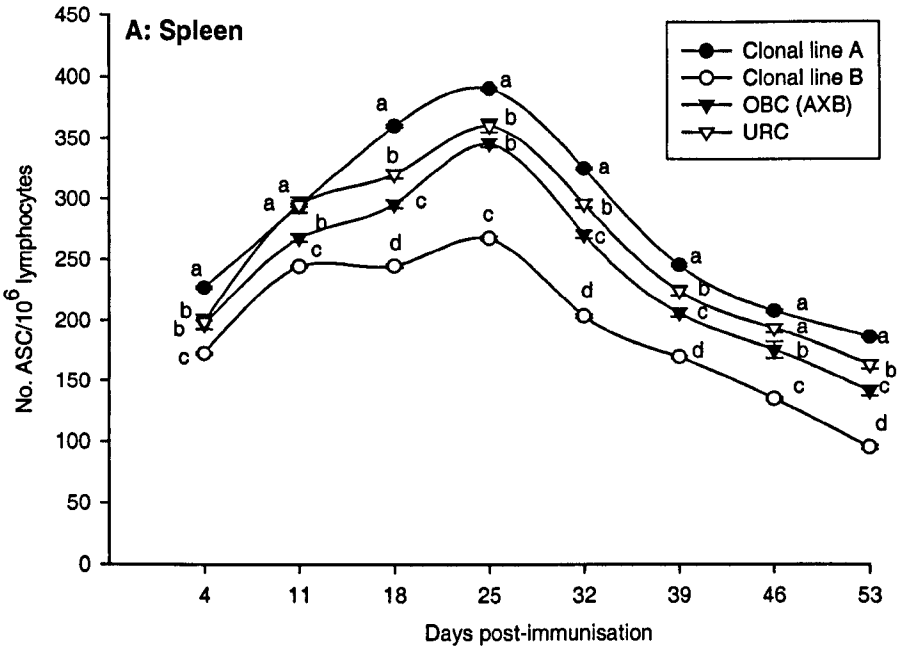
3.3.2. Immune response of Nile tilapia to thymus-dependent antigen (DNP-KLH)

3.3.2.1. Quantification of anti-DNP antibody secreting cells (ASC) in experimental groups of Nile tilapia

The kinetics of induced ASC followed a similar pattern in all groups of experimental fish but the number of ASC, which developed was different between the groups.

A rapid increase was observed in the number of ASC in both spleen and head kidney of all experimental groups of fish after immunisation, and all groups showed a peak level of ASC on day 25 post-immunisation. The number of ASC decreased in all experimental groups of fish after this time (Fig. 3.5).

The kinetics of the development of ASC in the different organs in the experimental groups of fish are shown in Fig. 3.5. Clonal line A fish had significantly ($P<0.05$) higher levels of ASC in both their spleen and their head kidney compared to clonal line B fish and OBC (AXB) fish at all times over the period of study. The number of ASC was higher in clonal line A fish than in URC fish and the difference was significant ($P<0.05$) on days 4, 18, 25, 32, 39 and 53 post-immunisation in spleen and on days 4, 18, 25, 39 and 53 post-immunisation in head kidney. Significantly ($P<0.05$) lower numbers of ASC were observed in the spleen and head kidney in clonal line B fish than those of OBC (AXB) and URC at all times over the period of study. Significantly ($P<0.05$) higher numbers of ASC were observed in URC fish than in OBC (AXB) fish in both spleen and head kidney over the period of study except on days 4 and 25 post-immunisation (Fig. 3.5).



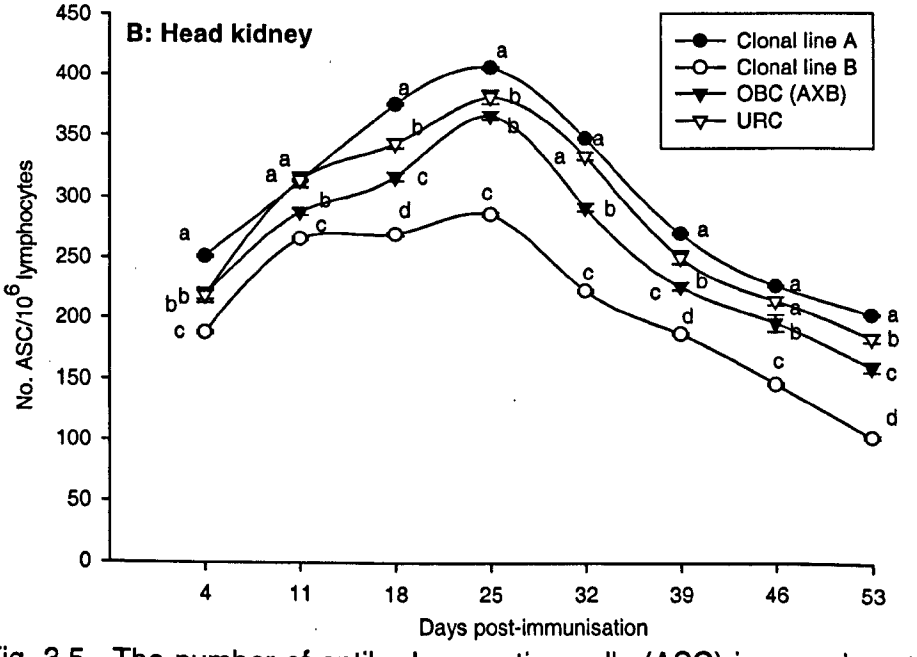


Fig. 3.5. The number of antibody secreting cells (ASC) in experimental groups of Nile tilapia (*Oreochromis niloticus*) after immunisation with dinitrophenyl-keyhole limpet haemocyanin (DNP-KLH), A: Spleen, B: Head kidney. Data showing comparison among different groups on the same day and are presented as mean ± SE ASC/10^6 lymphocytes of triplicate wells (N=5); different lower case letters indicate significant ($P<0.05$) difference.

The number of ASC in the different tissues of the same group of fish on same sampling day after immunisation with DNP-KLH over the study period is shown in Table 3.2.

Head kidney produced significantly ($P<0.05$) higher numbers of ASC than spleen except on days 18, 25 and 53 post immunisation in clonal line A fish. In clonal line B fish, head kidney ASC were significantly ($P<0.05$) higher in number than those of spleen on days 4, 18, 25 and 32 post-immunisation. The head kidney of OBC (AXB) and URC fish produced significantly ($P<0.05$) higher numbers of ASC than spleen ASC at all times over the period of study (Table 3.2).

Table 3.2. The number of antibody secreting cells (ASC) in different experimental groups of Nile tilapia after immunisation with dinitrophenyl-keyhole limpet haemocyanin (DNP-KLH). Data are presented as mean ± SE ASC/10^6 lymphocytes from triplicate wells (n=5). Different lower case letters indicate significant ($P<0.05$) differences between different tissues on same day; DPI: days post-immunisation.

Experimental Groups	DPI	Tissues	
		Spleen	Head kidney
		Mean ± SE	Mean ± SE
Clonal line A	4	227.2 ± 1.0 ^{b}	251.2 ± 1.0 ^{a}
	11	295.2 ± 1.2 ^{b}	313.2 ± 0.8 ^{a}
	18	360.4 ± 1.3 ^{a}	375.2 ± 1.0 ^{a}
	25	390.4 ± 1.2 ^{a}	405.6 ± 1.0 ^{a}
	32	325.2 ± 1.0 ^{b}	347.2 ± 1.0 ^{a}
	39	246.0 ± 1.3 ^{b}	269.6 ± 1.2 ^{a}
	46	208.4 ± 1.2 ^{b}	226.4 ± 0.7 ^{a}
	53	186.8 ± 0.8 ^{a}	202.8 ± 0.8 ^{a}
Clonal line B	4	172.4 ± 1.2 ^{b}	188.0 ± 1.1 ^{a}
	11	244.8 ± 1.0 ^{a}	265.2 ± 1.2 ^{a}
	18	244.8 ± 1.5 ^{b}	268.8 ± 1.5 ^{a}
	25	267.6 ± 1.5 ^{b}	285.6 ± 1.5 ^{a}
	32	203.6 ± 1.5 ^{b}	222.4 ± 1.2 ^{a}
	39	170.0 ± 1.1 ^{a}	187.2 ± 1.0 ^{a}
	46	135.2 ± 1.2 ^{a}	146.0 ± 1.1 ^{a}
	53	96.0 ± 1.5 ^{a}	103.2 ± 1.5 ^{a}
OBC (AXB)	4	196.4 ± 3.5 ^{b}	218.8 ± 3.6 ^{a}
	11	268.4 ± 3.1 ^{b}	287.6 ± 2.7 ^{a}
	18	295.8 ± 3.1 ^{b}	316.0 ± 3.3 ^{a}
	25	345.6 ± 3.4 ^{b}	366.0 ± 3.5 ^{a}
	32	270.8 ± 3.1 ^{b}	291.2 ± 3.3 ^{a}
	39	206.8 ± 3.3 ^{b}	226.0 ± 3.2 ^{a}
	46	176.0 ± 6.7 ^{b}	195.6 ± 6.8 ^{a}
	53	142.0 ± 4.1 ^{b}	160.0 ± 4.1 ^{a}
URC	4	198.8 ± 5.9 ^{b}	218.4 ± 5.9 ^{a}
	11	295.2 a 6.2 ^{b}	313.6 ± 5.6 ^{a}
	18	320.4 ± 3.5 ^{b}	342.8 ± 3.7 ^{a}
	25	360.4 ± 5.3 ^{b}	380.8 ± 5.5 ^{a}
	32	296.0 ± 3.2 ^{b}	332.4 ± 3.0 ^{a}
	39	224.8 ± 3.9 ^{b}	250.0 ± 5.2 ^{a}
	46	194.0 ± 3.6 ^{b}	214.0 ± 3.4 ^{a}
	53	164.0 ± 3.4 ^{b}	184.0 ± 3.4 ^{a}

No ASC were detected in either tissue of non-immunised fish in any of the experimental groups of fish used in the study.

An example of spots generated in the ELISPOT assay in this study in clonal lines A and B, OBC (AXB) and URC fish is presented in Fig. 3.6.

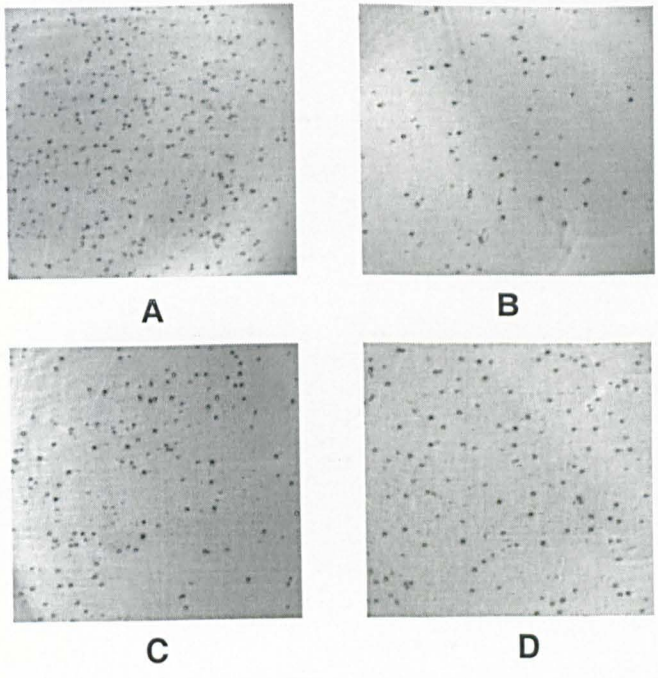


Fig. 3.6. A typical well showing spots generated in the ELISPOT assay. Spots indicate anti-DNP antibody secreting cells (ASC) 25 days post-immunisation with DNP-KLH in A: clonal line A, B: clonal line B, C: OBC (AXB), D: URC

3.3.2.2. Measurement of anti-DNP serum antibody titre in Nile tilapia experimental groups of fish

The kinetics of the level of serum antibody against DNP followed similar patterns in all experimental groups of fish but the level of these titres differed between groups (Fig. 3.7).

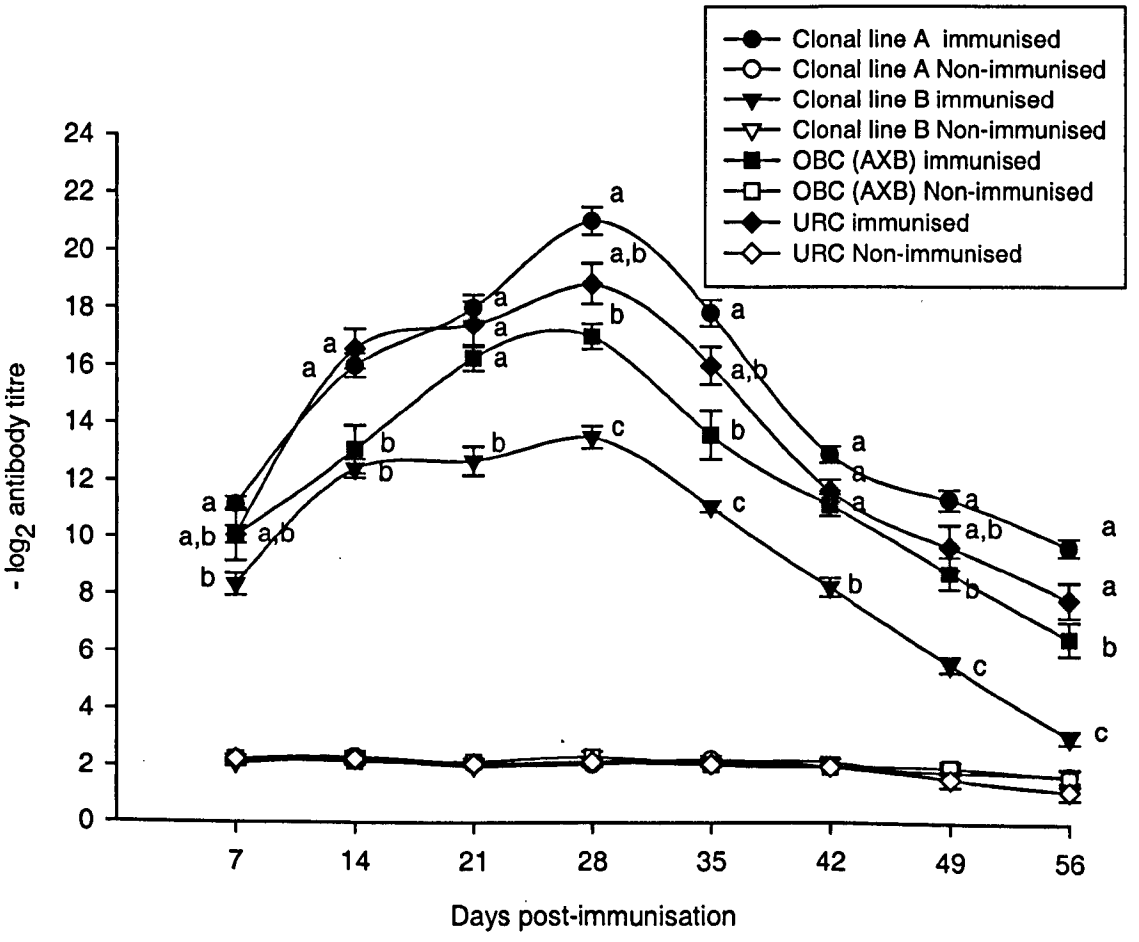


Fig. 3.7. Serum anti-DNP antibody titre in different experimental groups of Nile tilapia (*Oreochromis niloticus*) following immunisation with dinitrophenyl-keylole limpet haemocyanin (DNP-KLH). Data are presented as mean ± SE of $-\log_2$ titre (N=12), compared among different groups on the same day; different lower case letters indicate significant ($P<0.05$) difference.

All experimental groups of fish had peak antibody titres on day 28 post-immunisation. After that, the antibody titre of all experimental groups of fish started to

decline. Very low antibody titres were observed in clonal line B fish by day 56 post-immunisation (Fig. 3.7).

The antibody titres of clonal line A fish were significantly ($P<0.05$) higher than titres of clonal line B fish at all times over the period of study and were also significantly ($P<0.05$) higher than the titre of OBC (AXB) fish except on days 7, 21 and 42 post-immunisation. URC fish had lower antibody titres than clonal line fish A but these were not significantly different (Fig. 3.7).

Clonal line B fish had lower antibody titres than the titres of both OBC (AXB) fish and URC fish but the differences in titre between clonal line B and OBC (AXB) fish and clonal line B and URC fish were significant ($P<0.05$) from day 21 and day 14 post-immunisation, respectively (Fig. 3.7).

The URC fish had higher titres than OBC (AXB) fish but this difference was only significant ($P<0.05$) on days 14 and 56 post-immunisation (Fig. 3.7).

The non-immunised fish of clonal line A and B, OBC (AXB) and URC had very low titres due to a non-specific response against the antigen and no difference was observed in the titres between the different groups of fish (Fig. 3.7). Mean anti-DNP titre of the experimental groups of fish are also presented in Appendix 2.

3.3.3. Immune response of Nile tilapia to thymus-Independent antigen (TNP-LPS)

3.3.3.1. Quantification of anti-TNP antibody secreting cells (ASC) in experimental groups of Nile tilapia

The kinetics of the appearance of ASC in spleen and head kidney following immunisation of fish with TNP-LPS was similar between the different experimental groups of fish and as seen with the response with the response against SRBC and DNP-KLH, the numbers of ASC present differed between the experimental groups of fish. Fig. 3.8 represents the kinetics of the appearance of ASC in the different tissues of experimental groups of fish following immunisation with TNP-LPS. The development of ASC was very quick in all experimental groups of fish and the number of ASC increased rapidly reaching peak response on day 14 post-immunisation. After this peak response, the number of ASC number started to decline in all experimental groups of fish (Fig. 3.8).

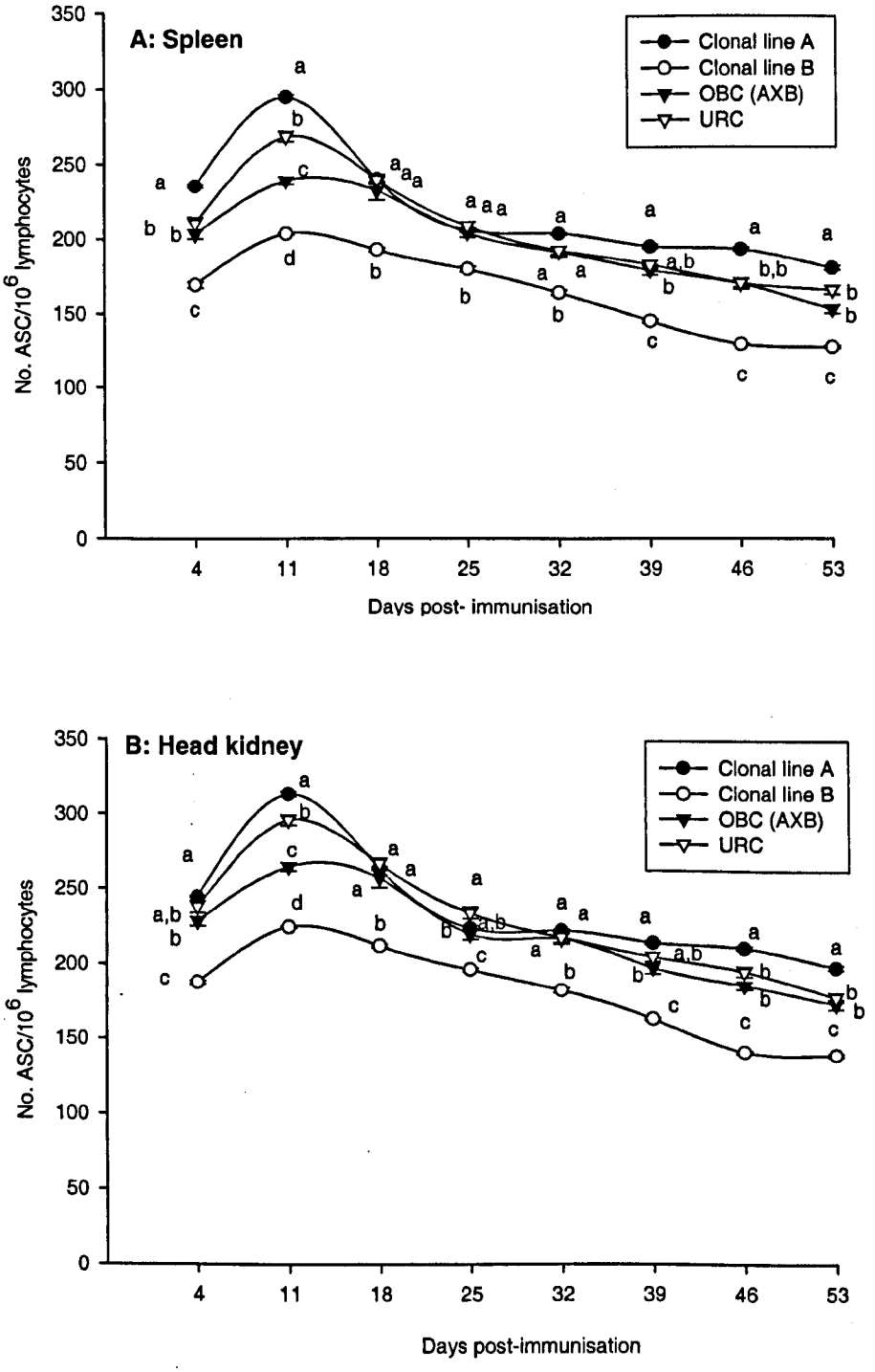


Fig. 3.8. Number of ASC in A: Spleen, B: Head kidney of different experimental groups of Nile tilapia (*Oreochromis niloticus*) after immunisation with trinitrophenyl-lipopolysaccharides (TNP-LPS). Data showing comparison among different groups on the same day and are presented as mean ± SE ASC/10^6 lymphocytes of triplicate wells (N=5); different lower case letters indicate significant ($P<0.05$) difference.

Clonal line A fish had a significantly ($P<0.05$) higher numbers of ASC in both their spleen and their head kidney than clonal line B fish at all times post-immunisation over the period of study. Significantly ($P<0.05$) higher numbers of ASC were also observed in clonal line A fish than OBC (AXB) fish in both their spleen and kidney on days 4, 11, 39 to 53 post-immunisation. Clonal line A fish had a significantly ($P<0.05$) higher number of ASC than URC fish on days 4, 11, 46 and 53 post-immunisation in spleen and on days 11, 46 and 53 post- immunisation in head kidney (Fig. 3.8).

Significantly ($P<0.05$) lower numbers of ASC were observed in the spleen and head kidney in clonal line B fish than those of OBC (AXB) and URC at all times examined post-immunisation (Fig. 3.8).

Significantly ($P<0.05$) higher numbers of ASC were observed in the spleen and head kidney of URC fish than the numbers in OBC (AXB) fish on day 11 post-immunisation (Fig. 3.8).

The number of ASC in different tissues in the same group of fish on same sampling day after immunisation with TNP-LPS are presented in Table 3.3.

Table 3.3. The number of antibody secreting cells (ASC) in different experimental groups of Nile tilapia after immunisation with trinitrophenyl-lipopolysaccharides (TNP-LPS). Data are presented as mean ± SE ASC/10^6 lymphocytes from triplicate wells (n=5). Different lower case letters indicate significant ($P<0.05$) differences between different tissues on same day; DPI: days post-immunisation.

Experimental Groups	DPI	Tissues	
		Spleen	Head kidney
		Mean ± SE	Mean ± SE
Clonal line A	4	235.6 ± 1.0 [a]	244.4 ± 0.7 [a]
	11	294.8 ± 1.4 [b]	313.2 ± 1.6 [a]
	18	240.0 ± 1.7 [b]	263.2 ± 1.0 [a]
	25	206.0 ± 1.4 [b]	223.2 ± 1.6 [a]
	32	203.6 ± 0.7 [b]	221.6 ± 0.7 [a]
	39	194.8 ± 1.2 [b]	213.6 ± 1.2 [a]
	46	192.8 ± 1.2 [b]	210.8 ± 1.2 [a]
	53	180.4 ± 1.5 [b]	198.4 ± 1.5 [a]
Clonal line B	4	169.6 ± 1.9 [b]	187.6 ± 1.2 [a]
	11	203.6 ± 0.7 [b]	224.4 ± 0.7 [a]
	18	192.8 ± 1.2 [b]	212.0 ± 0.9 [a]
	25	180.0 ± 1.7 [b]	196.0 ± 1.1 [a]
	32	164.4 ± 1.6 [b]	182.0 ± 1.7 [a]
	39	145.2 ± 1.0 [b]	163.2 ± 0.8 [a]
	46	129.0 ± 0.4 [a]	141.2 ± 1.0 [a]
	53	127.2 ± 0.8 [a]	139.6 ± 1.2 [a]
OBC (AXB)	4	203.2 ± 3.0 [b]	228.0 ± 2.8 [a]
	11	239.2 ± 2.4 [b]	264.4 ± 2.8 [a]
	18	232.4 ± 6.2 [b]	256.8 ± 6.3 [a]
	25	204.0 ± 2.8 [b]	219.2 ± 3.4 [a]
	32	191.6 ± 3.3 [b]	216.4 ± 3.2 [a]
	39	179.2 ± 3.3 [b]	197.2 ± 3.9 [a]
	46	170.0 ± 3.5 [b]	186.0 ± 2.6 [a]
	53	152.8 ± 2.9 [b]	174.0 ± 3.2 [a]
URC	4	210.4 ± 4.0 [b]	237.6 ± 3.9 [a]
	11	268.8 ± 3.3 [b]	295.6 ± 3.4 [a]
	18	240.0 ± 3.0 [b]	266.4 ± 3.4 [a]
	25	208.8 ± 2.4 [b]	233.2 ± 3.7 [a]
	32	192.4 ± 2.6 [b]	217.2 ± 3.4 [a]
	39	182.8 ± 2.7 [b]	204.4 ± 3.0 [a]
	46	170.4 ± 2.9 [b]	195.2 ± 3.3 [a]
	53	165.8 ± 2.9 [a]	178.4 ± 3.0 [a]

Head kidney appeared to have a higher number of ASC than spleen but the difference is significant ($P<0.05$) from day 11 to 53 post-immunisation in clonal line A, from day 4 to 39 in clonal line B fish and at all times over the period of study in OBC (AXB) fish and from day 4 to 46 in URC (Table 3.3).

No non-immunised fish from any group appeared to produce ASC in either spleen or head kidney.

3.3.3.2. Measurement of anti-TNP serum antibody titre in Nile tilapia experimental groups of fish

The kinetics of serum antibody titre, which developed after immunisation with TNP-LPS in different groups of experimental fish is presented in Fig. 3.9. Serum antibody titres followed similar pattern of kinetics in each group over the course of the study but they differed in magnitude. Peak antibody titres were observed on day 14 post-immunisation in all groups of experimental fish.

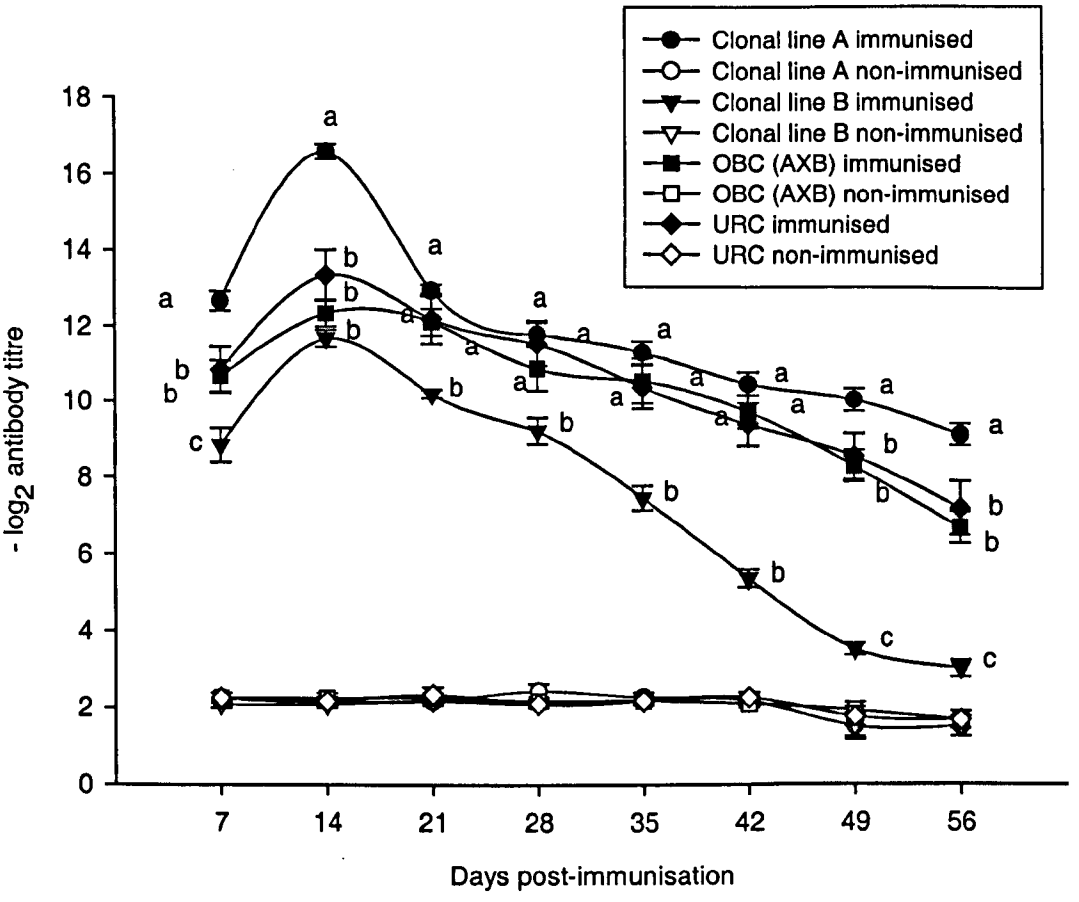


Fig. 3.9. Serum anti-TNP antibody titre in different experimental groups Nile tilapia (*Oreochromis niloticus*) following immunisation with trinitrophenyl-lipopolysaccharides (TNP-LPS). Data are presented as mean ± SE of -$\log_2$ titre (N=12), compared among different groups on the same day; different lower case letters indicate significant ($P<0.05$) difference.

The antibody titres measured in clonal line A fish were significantly ($P<0.05$) higher than the titre obtained from clonal line B fish at all times over the period of study. Clonal line A fish also had a higher titre than both OBC (AXB) and URC fish but these were only significantly ($P<0.05$) different on days 7, 14, 49 and 56 post-immunisation. The titres of clonal line B fish were significantly ($P<0.05$) lower than the titres of OBC (AXB) and URC fish except on day 14 post-immunisation. OBC (AXB) and URC fish appeared to have antibody titres with values between the titres of clonal line A and

clonal line B fish, but the titres between these two groups were not significantly different from each other (Fig. 3.9).

The non-immunised fish of clonal line A and B, OBC (AXB) and URC had a very low non- specific antibody titre and no difference was observed in this titre among the different groups of fish. Mean anti-TNP serum antibody titres of the experimental groups of fish are also presented in Appendix 3.

3.4. Discussion

The aim of the experiment was to compare the specific immune response of the different experimental groups of Nile tilapia to a variety of different test antigens. The results of the specific immune responses of two Nile tilapia inbred clonal lines (clonal line A and B), one outbred clonal line OBC (AXB) and unrelated control group (URC) examined in this chapter are discussed here.

The specific immune response of the experimental fish was measured following immunisation with test antigens SRBC, DNP-KLH, TNP-LPS. The kinetics of development of ASC in different lymphoid tissues was assessed by a PFC assay or an ELISPOT assay using lymphocytes from blood, spleen and head kidney. Serum antibody titre was measured by an ELISA following immunisation.

All experimental fish had a similar pattern in the kinetics of the development of PFC or ASC responses and antibody titres to SRBC, DNP-KLH and TNP-LPS but the magnitude of these responses differed between experimental groups. In general, the specific immune response of clonal line A fish was always significantly higher than that of clonal line B fish. The OBC (AXB) line fish had an intermediate response between clonal lines A and B fish which was generally significantly lower than clonal line A fish and significantly higher than that of clonal line B fish in most but not all cases. The response of URC fish was lower than that of clonal line A, but this group was generally not significantly different to clonal line A with a few exceptions. The responses observed between OBC (AXB) line and URC fish were very similar.

A number of studies have focused on divergent selection for antibody production. Some of the best known examples are with Swiss albino mice by Biozzi *et al.* (1979)

and in chickens (Van der Zijpp and Nieuwland, 1986). Mice were initially selected for high and low antibody production to SRBC. The profound differences between high and low responder mice showed a similar response to a wide range of antigens (various heterologous erythrocytes, proteins, haptens, bacterial polysaccharides, histocompatibility and tumor antigens, viruses, bacteria and parasites) with the sole exception of certain polysaccharides (Biozzi *et al.*, 1971; Howard *et al.*, 1974). These mice lines have shown little or no interline differences in various manifestations of T-cell mediated immunity i.e. skin allograft rejection and cytotoxic antibody production (cited in Kierszenbum and Howard, 1976). The quantitative differences in antibody responsiveness were ascribed to genetic modifications in macrophage metabolism and antigen presentation as well as to quantitative differences in B cell subsets (Biozzi *et al.*, 1984). The high rate of macrophage metabolism in the low responder lines suggested an inverse relationship between genetic regulation of macrophage activity and antibody responsiveness (Biozzi *et al.*, 1984).

A two-way selection procedure initiated by Siegel and Gross (1980) resulted in different antibody response to both T-dependent and T-independent antigens (Scott *et al.*, 1994). Van der Zijpp and Nieuwland (1986) also initiated a divergent selection for anti-SRBC response in chicken. These chicken lines had similar responses to bovine serum albumin, BSA (Parmentier *et al.*, 1994). Studies on subsequent generations of these high and low responder chickens revealed no differences with respect to phagocytic activity but did show that the selection had affected both the cellular responses (Kreukniet *et al.*, 1994), and the number of various T cell subsets and B cells (Parmentier *et al.*, 1995). It was concluded that antigen processing and presentation but

not phagocytosis had determined the differences in antibody responses between the chicken lines (Parmentier *et al.*, 1994). Van der Zijpp and Nieuwland (1986) chickens selected for high and low antibody response did not differ in macrophage ability to respond to antigenic challenge (reviewed by Scott *et al.*, 1994).

Similarly to studies on mice (Biozzi *et al.*, 1979) and chickens (Van der Zijpp and Nieuwland, 1986), common carp were divergently selected for high or low antibody response to DNP-KLH (Wiegertjes *et al.*, 1994). Similar to the observed difference in response to DNP-KLH, immunisation with TNP-LPS also induced a difference in antibody production between the high and low responder lines. The authors suggested that the antibody production after TNP-LPS immunisation might be a measure of the size of the nitrophenyl-sensitive precursor pool of B cells which may have changed owing to selection. The magnitude of antibody production in F1 progeny from a cross between high and low responder lines suggested that high responsiveness to DNP-KLH was inherited in a dominant fashion. The antibody production to another T-dependent antigen dinitrophenyl-human serum albumin DNP-HSA, did not always follow the same pattern of response to DNP-KLH. These differences might be due to at least the carrier or T cell dependency in responsiveness (Wiegertjes *et al.*, 1995a).

Differences in the specific response between the experimental groups of Nile tilapia may be correlated to the number of respective antigen specific B cells (Wiegertjes *et al.*, 1995a). High responding clonal line A may possess a higher number of respective antigen-specific B cells. This would result in a difference in the number of ASC produced by different groups of experimental fish as well as subsequent difference in serum antibody titre as described in divergently selected chicken lines (Kreukniet *et al.*,

1992). The differences in antibody producing capacity cannot be solely attributed to a larger number of B cells that reacts to antigens. In addition to specific immunity, adjuvant stimulates cell-mediated immunity (Allison and Byars, 1986) such as T cell activity some of which are T helper population (Allison and Davies, 1971; Waldman and Pope, 1977). Accessory cells are also probably stimulated and release factors like interleukin-1 which stimulates the proliferation of helper T cells. Due to T cell help enhanced by Complete Freund's Adjuvant (CFA), divergently selected lines can produce antibodies equally well, independent of the number of antigen specific B cells (Kreukniet *et al.*, 1992). Differences in specific response in the different experimental fish groups might be caused by differences in the T cell activity as suggested for chicken by Kreukniet *et al.* (1992).

In the present study, antibody production after immunisation may be a measure of the size of the respective antigen sensitive B cell precursor pools as observed in common carp (Wiegertjes *et al.*, 1995a). Limiting dilution assays of lymphocytes taken from organs of immunised fish may detect differences in antigen sensitive precursor pool frequencies in different experimental groups of fish, allowing further characterisation of high and low responding clonal lines as suggested by Wiegertjes *et al.* (1995a).

The culture system used to measure the generation of ASC *in vitro*, has been widely used in endothermic vertebrates. The method was originally described for PFC assay (Mishell and Dutton, 1966). The PFC assay has proven to be an easy and effective tool for detecting and quantifying ASC *in vitro*. This assay system had also been used to detect specific antibody secreting cells from a number of fish species such as in Mozambique tilapia (Sailendri and Muthukkaruppan, 1975a), rainbow trout (Anderson

et al., 1979; Georgopoulou and Vernier 1986; Findlay, 1994), channel catfish (Miller and Clem, 1984), air breathing catfish *Clarius batrachus* (Sinha and Chakravarty, 1997) and common carp (Rijkers *et al.*, 1980). A more sensitive assay for the enumeration of ASC, the Enzyme Linked Immunosorbent (ELISPOT) assay has since been developed based upon immuno-enzyme technology (Czerkinsky *et al.*, 1983; Sedgwick and Holt, 1983). Utililization of PFC assay has been limited by the obligatory use of lysable SRBC as indicator of antibody secretion, the necessity of coupling antigens to SRBC, the presence of natural haemolysins in fish serum to SRBC and the intensive labour and time required to conduce the assay. ELISPOT assay has removed many of the constraints associated with PFC assay, has afforded an equal or greater sensitivity and a reduction in both time and labour. Adaptation of this of this assay for use in fish has significantly improved the ability to assess immune responses among fish (Waterstrat *et al.*, 1991)

The ELISPOT assay was used in this study to enumerate the number of ASC in fish immunised with TD (DNP-KLH) and TI (TNP-LPS) antigens. This assay was easy to set up and consistently produced reproducible results. This methodology has already been used to detect ASC in fish such as channel catfish (Waterstrat *et al.*, 1991), dab *Limanda limanda* (Secombes *et al.*, 1991), rainbow trout (Davidson *et al.*, 1992, 1993; Siwicki and Dunier, 1993), roach *Rutilus rutilus* (Aaltonen *et al.*, 1994) and common carp (Koumans-van Diepen *et al.*, 1995; Bandin *et al.*, 1997) immunised with different antigens.

Earlier reports showed that the major organs for antibody secretion in Nile tilapia are head kidney and spleen while peripheral blood can be regarded as the minor organ with

fewer ASC present after immunisation (Sailendri and Muthukkaruppan, 1975a). In the present study, peripheral blood lymphocytes produced significantly fewer numbers of PFC than head kidney and spleen lymphocytes when SRBC was used as antigen. In all groups of experimental fish, head kidney produced significantly higher numbers of PFC and ASC against SRBC, DNP-KLH and TNP-LPS than other tissues. Surprisingly no ASC were detected in the peripheral blood lymphocytes against DNP-KLH and TNP-LPS. Earlier studies on air breathing catfish (Sinha and Chakravarty, 1997), channel catfish (Miller and Clem, 1984) and common carp (Rijkers *et al.*, 1980; Bandin *et al.*, 1997) have reported head kidney to produce the highest number of ASCs. Miller and Clem (1984) found that head kidney generated the highest number of PFC while spleen and peripheral blood lymphocytes gave 10% fewer plaques in channel catfish. While, Bandin *et al.* (1997) reported low numbers of ASC in spleen lymphocytes compared to head and trunk kidney with head kidney producing the best results in common carp.

Some of the major factors affecting the number of antibody secreting cells in PFC and ELISPOT assays appear to be the incubation period and the concentration of lymphocytes used in culture, as well as the source of serum which is the source of complement in the PFC assay. In the present study, the incubation period of lymphocytes was kept constant for a certain assay (five hours in PFC assay and nine hours in ELISPOT assay). Therefore, the differences in the final counts of PFC and ASC obtained between different groups of experimental fish were not due to differences in the incubation period. The results revealed that spots clearly appeared after incubation for 9 hours in the ELISPOT assay. In common carp, Bandin *et al.* (1997) also used 9 hours of incubation time as optimum after series of trials to optimise

incubation time from four hours to 18 hours. In the present experiment no such experiment was conducted to optimise the incubation time for the ELISPOT assay. Therefore, the number of spots formed in relation to the duration of incubation period could not be interpreted. However, in several studies it was found that after four hours of incubation some plaques could be observed although they were small in size, not clearly defined and relatively low in number. After six hours the plaques were readily discernible and this number remained constant when incubation time increased to 18 hours after which the plaque gradually faded. These plaques were large, clearly developed and easily enumerated. Shorter incubation periods (three to five hours) resulted in fainter/less defined spots that were often much lower in number as observed by Secombes *et al.* (1991) in dab. Most likely, the concentration of antibodies produced by ASC after three to five hours incubation was too low to be clearly detected. Longer incubation periods (more than nine hours) resulted in lower number of spots being developed and it was suggested that this could be due to the dissociation of secreted low affinity antibody (Secombes *et al.*, 1991; Bandin *et al.*, 1997).

In order to determine the concentration of lymphocytes required for optimal responses in PFC and ELISPOT assays *in vitro*, a number of different cell concentrations were tested in the present study and finally $5x10^5$ cells per well in 96 well cell culture plates was used in both assays to eliminate differences in the number of final counts of spots due to differences in the concentration of lymphocytes cultured in the assays. Miller and Clem (1984) and Findlay (1994) have reported that at lower concentrations of lymphocytes the number of PFCs which resulted were lower than expected. Although the reasons for the disproportionate drop in the numbers of PFC

generated is unclear, according to Miller and Clem (1984) this may be due to the reduced cell-cell contact at lower concentration resulting in less efficient cellular interactions as routinely seen in cultures of cells from higher vertebrates (Mishell and Dutton, 1966). Miller and Clem (1984) used 1 $x10^6$ cells $well^{-1}$ in channel catfish and Findlay (1994) used 2.5 x 10^5 cells per well in rainbow trout.

Miller and Clem (1984) in their study on channel catfish and Sinha and Chakrabarty (1997) on air-breathing catfish suggested using guinea pig serum as a complement source. Naive catfish serum contains an effective natural haemolysin for SRBC and this haemolytic activity often precludes its use as a complement source for the PFC assay. This has also been reported in trout serum (Mishell and Shiigi, 1980). Rijkers *et al.* (1980a) obtained their best results in carp when bream *Abramis brama* serum was used as the source of complement. Mozambique tilapia serum was used as the complement source as used by Sailendri and Muthukkaruppan (1975a). Findlay (1994) also used homologous rainbow trout serum. However, the homologous serum was absorbed against SRBC before using it in the PFC assay, to remove naturally occurring antibodies, which would otherwise lyse the SRBC (Sailendri and Muthukkaruppan, 1975a). Differences in the PFC response observed in the present experiment were not due to different complement sources in the assay since pooled serum was kept frozen after absorbing against SRBC and the same batch was used for each PFC assay.

In the present study, the spots in ELISPOT assay seemed more intense in clonal line A fish than the clonal line B fish. Bandin *et al.* (1997) who studied ASC in high and low responder carp strains suggested this could indicate a qualitative difference between the ASC obtained from high and low responder clonal lines. This might be related to

differences in the affinity of antibodies produced by the ASC (Secombes *et al.*, 1991) which might be detectable in the serum of high and low responder strain using an ELISA based technique (Kaattari and Shapiro, 1994).

In the present experiment serum antibody titres were measured by ELISA after immunisation with different antigens. Different groups of experimental fish had different levels of antibody titre after immunisation with different antigens. The profile of the antibody titre over the course of the study followed the pattern of ASC, with slight irregularities, when in some sampling days post-immunisation, significant difference in ASC response did not result in significant difference in antibody titre between experimental groups. Although, in general, the number of ASC determines the subsequent serum antibody response, high versus low differences were most obvious comparing the number of ASC and not antibody production (Bandin *et al.*, 1997). It has been suggested that serum antibody levels, being the final product of the immune response, reflect larger environmental variation than the number of ASC does. The reason for this may be because persisting antigen may bind circulating antibodies or the accumulation of antibodies in the serum is likely to conceal a direct correlation of the numbers of ASC measured from different lymphoid organs. Serum antibody titres can remain at an elevated level after immunisation when ASC response returns to background level (Sinha and Chakravarty, 1997). This may be the reason behind significant difference in ASC but non-significant difference in antibody titre at the corresponding time between experimental groups of the present experiment.

It is well documented that temperature or season has profound effects on the immune response of fish, with low temperature suppressing their immune response (Avtalion *et*

al., 1973). Antibody production and immunological memory can also be dissociated in carp when the animals are primed at temperatures below 15°C (Avtalion, 1969). In these conditions a three to four day delay is necessary to generate memory cells but no antibodies are secreted. Putting the fish at 25°C allows antibody secretion (Desvaux and Charlemagne, 1981). Sinha and Chakravarty (1997) also reported a seasonal effect on ASC in air breathing catfish with fewer ASC produced in winter than in summer. Rainbow trout showed an important individual variability in specific immune response when immunisations were done in similar conditions but at different times of the year (Cossarini-Dunier, 1986). In the present study, all experimental fish were reared and maintained at the same temperature, optimum for Nile tilapia (27° ± 1°C). Therefore, any variation in the immune response of fish in this study due to temperature variation can be ruled out.

The immunisation route and dose are known to affect on the specific immune response of fish (Lamers *et al.*, 1985; Neumann and Tripp, 1986; Ellis, 1988; Jones *et al.*, 1993). In the present study, all experimental fish were immunised by i.p injection of antigen and dose of antigen was either optimised prior to the study in preliminary trials or followed doses outlined in published reports. All fish received the same dose of a particular antigen. Processes that stimulate antibody response when antigens entered the body, may be more efficient in the higher responding clonal line A than clonal line B, with OBC (AXB) having intermediate efficiency and URC almost similar efficiency to clonal line A.

In this study, a soluble form of TD hapten-carrier conjugate (DNP-KLH) was used as an antigen, which might not have resulted in optimum primary specific response in the

fish. The form of hapten (soluble or particulate) has been shown to affect the magnitude of the primary and secondary ASC responses when a hapten-carrier conjugate is used as a antigen (Miller and Clem, 1984). In channel catfish, the form of the antigen had little effect on the magnitude of the primary anti-hapten response to TI antigen and also on the secondary response to T-dependent antigen. However, the generation of primary anti-hapten response to TD antigen was critically dependent on the form of the antigen form, i.e., particulate TD antigen was necessary in order to obtain an optimal PFC response (Miller and Clem, 1984), which was also observed in murine cells (Katz, 1977). The primary aim of this study was to find out how the different groups of experimental fish responded in terms of their specific immune response to different types of antigen. Identical patterns in magnitude of specific response to the soluble form of TD antigen were observed in different groups of experimental fish, as seen with the other antigens used in the study.

Quantitative traits associated with reproduction or physiological efficiency such as growth and survival are often affected by inbreeding depression, which in turn might improve in hybrid lines (Kincaid, 1983). In the present study, OBC (AXB) fish showed intermediate response, significantly lower response than clonal line A and significantly higher response than clonal line B after immunisation with hapten-carrier conjugates DNP-KLH and TNP-LPS. This indicated additive parental effects on OBC (AXB) fish. When SRBC was used, the response of OBC (AXB) was significantly higher than clonal line B but not significantly different from the response of clonal line A, indicating a dominant inheritance of higher responsiveness in OBC (AXB) fish to this antigen. The reason for this cannot readily be explained.

From the above discussion it is revealed that clonal line A had significantly higher specific immune responses than clonal line B fish to all three test antigens used in the study. Any difference in specific immune response in the two clonal lines should be due to genetic differences between the two fully inbred clonal lines of Nile tilapia. The specific immune response of the clonal lines A and B to test antigens might have potential as an indirect marker for selection for disease resistance, if survival after exposure to pathogens has positive correlation with specific immune response in the respective clonal lines.

CHAPTER 4

SPECIFIC IMMUNE RESPONSE AND DISEASE RESISTANCE OF CLONAL LINES IN NILE TILAPIA *Oreochromis niloticus* TO *Aeromonas hydrophila* T4

4.1. Introduction

Species or populations with resistance to disease are important candidates for selection (Fjalestad *et al.*, 1993). The advantage of indirect selection criteria for disease resistance such as immune response parameters is that the difficult experimental protocols involving exposing fish to pathogens necessary for direct selection can be avoided (Chevassus and Dorson, 1990).

Disease resistance can be recorded by exposing the fish to specific pathogens (Van Muiswinkel *et al.*, 1990; Gjedrem *et al.*, 1991; Ibarra *et al.*, 1991; Refstie *et al.*, 1993 cited by Van Muiswinkel, 1999; Stromsheim *et al.*, 1994a,b; Eide *et al.*, 1994; Gjedrem and Gjoen, 1995). Although significant genetic variation has been found in immune responses, more knowledge is needed on the genetic correlation of immune parameters with disease resistance, before those parameters can be used as markers for indirect selection (Fjalestad *et al.*, 1993). Survival in experimental challenge and physiological and immunological parameters are often used together as indicators of immune status and disease resistance (Biozzi *et al.*, 1984; Cipriano and Heartwell, 1986; Hamilton *et al.*, 1986; Fevolden *et al*, 1991, 1992, 1994; Balfry *et al.*, 1994; Gjedrem *et al.*, 1991; Gjedrem and Gjoen, 1995; Lund *et al.*, 1995b; Wiegertjes *et al.*, 1995a,b; Slierendrecht *et al.*, 1993, 1996; Scott *et al.*, 1994).

Test antigens such as SRBC, DNP-KLH and TNP-LPS were used as tools to compare the specific immune responses of two clonal lines of Nile tilapia (clonal lines A and B), an outbred clonal line OBC AXB, and an unrelated control group, URC of Nile tilapia in Chapter-3. The use of well defined antigen determinants to select for immune responsiveness increases the detection level of genetic determination but may not have a

causative link with resistance or susceptibility to disease (Wiegertjes *et al.*, 1995a). Therefore a disease causing organism was used in this chapter to study the immune response as well as resistance/susceptibility of the experimental groups of Nile tilapia to disease.

Aeromonas hydrophila is considered to be the principal cause of bacterial haemorrhagic septicaemia in tilapia, channel catfish, carp, eel, milkfish *Chanos chanos*, ayu and trout (Amin *et al.*, 1985; Miyazaki and Jo, 1985; Frerichs, 1989; Rahman *et al.*, 1997). *A. hydrophila* is a ubiquitous and heterogeneous organism, which produces disease under stressed conditions or in concert with infection by other pathogens (Rahman and Kawai, 2000) and is associated with a variety of ulcerative conditions including epizootic ulcerative syndrome (EUS) caused by *Aphanomyces invadans* in south-east Asia (Llobrera and Gacutan, 1987; Lio-po *et al.*, 1992; Millar, 1994). Fish can be at risk at any time due to the ubiquitous distribution of the organism (Frerichs and Roberts, 1989). *A. hydrophila* is a suitable fish pathogen for artificially producing disease in Nile tilapia (Sarder, 1998). Therefore this disease-causing organism was chosen to study the immune response of the clonal Nile tilapia. The strain of *A. hydrophila* T4, used in this study, was isolated from Indian major carp, during an outbreak of epizootic ulcerative syndrome in Bangladesh (Millar, 1994).

4.1.1. The aim of the study

The aim of the experiment was to study and compare the specific immune responses and disease resistance of the different experimental groups of Nile tilapia, clonal line A; clonal line B; outbred clonal line OBC (AXB) and unrelated control (URC) to *A. hydrophila* T4.

4.2. Materials and methods

4.2.1. Experimental fish and their maintenance

The Nile tilapia species, used in the experiments came from the Tilapia Reference Collection at the Institute of Aquaculture, University of Stirling. Their production, propagation and maintenance were described in Chapter 2 and 3 of this thesis.

4.2.2. Optimisation of *A. hydrophila* T4 concentration for vaccine preparation

The concentration of *A. hydrophila* T4 to be used in vaccination was determined in a trial experiment. Initially three bacterial concentrations were used (1x 10^7 ml^{-1}, 1x10^8 ml^{-1} and 1x10^9 ml^{-1}) and 5 fish were used per vaccine dose. An i.p. injection of 0.2ml was administered to each fish. Fish were bled from the caudal vein over a period of 56 days post-vaccination. An enzyme-linked immunosorbent assay (ELISA) was carried out as described in Section 3.2.3.2 to determine the antibody titre of the serum samples. The results of optimisation trial are described in Section 4.3.1.1.

4.2.2.1. Vaccination of experimental fish against *A. hydrophila* T4

Heat killed *A. hydrophila* T4 with adjuvant was used to vaccinate the experimental fish. The bacteria were cultured in tryptone soya broth (TSB) for 24 hour at 22°C following removal from -70°C preservation. The broth culture was plated out onto a tryptone soya agar (TSA) plate to check the purity of the culture. The plate was used as a stock plate. One or two colonies of bacteria were removed from the plate and cultured in 100 ml of TSB overnight at 22°C. Bacteria were harvested by centrifuging at 800 g for 10 min. The resultant pellets were washed twice with sterile PBS and then finally

resuspended in 10 ml of sterile PBS. The bacterial suspension was heat killed in a water bath at 75°C for 15 min. A loop full of the heat-killed suspension was streaked onto a TSA plate and incubated overnight at 22°C to confirm heat killing. Before heat killing, a colony forming unit (CFU) count was performed. The absorbance of the prepared suspension was measured in spectrophotometer at 610 nm. The concentration of the prepared bacterial suspension was determined by relating the absorbance of the suspension at 610 nm to a pre-made standard curve for *A. hydrophila* T4, relating concentration to absorbance and also from CFU counts. This was used as the stock bacterial suspension and adjusted accordingly to make up a suspension of desirable bacterial concentration.

Vaccine was prepared as 3:7 (v/v) mixture of heat-killed bacteria in PBS and "Montanide" (Aquatic Vaccines Ltd., UK) to give final bacterial concentration of $1x10^8$ cells ml^{-1}, optimised in the trial experiment.

Seventy-eight fish from each experimental group were used. All fish were split into six tanks with 13 fish from each experimental group in the same tank. Fish from three tanks were vaccinated with heat killed *A. hydrophila* T4 and fish from the other three tanks were injected with PBS in adjuvant as the non-vaccinated control. Therefore, in total, 39 fish from each group were vaccinated with *A. hydrophila* T4 and 39 fish were kept as non-vaccinated control. Each vaccinated fish received an i.p. injection of 0.2ml of the prepared vaccine and non-vaccinated fish were injected with the same dose of PBS.

The fish were bled from the caudal vein at 14 day intervals on days 14, 28, 42 and 56 post-vaccination. Serum samples were collected and stored at -70°C until used in

ELISA.

4.2.2.2. Enzyme-Linked Immunosorbent Assay (ELISA)

An indirect ELISA was used to measure serum antibody titre against *A. hydrophila* T4, following the protocol described by Chen *et al.* (1996). The basic procedure (incubation time and washings) was the same as described in Section 3.2.3.2.

Ninety six well ELISA plates were coated with 1% poly-L-lysine in bicarbonate buffer (0.125 M carbonate- bicarbonate buffer, pH 9.6) and were incubated for 1 hour at room temperature. After washing, a heat-killed bacterial suspension in PBS of $1x10^8$ cells ml^{-1} was added to the wells at 100 µl per well and plates were incubated for 1.5 hours at 21°C. A 1:1000 dilution of 50% glutaraldehyde was made in PBS (pH 7.2, 0.02M) and 50 µl was added to each well for 20 min to fix the bacteria. Plates were blocked by adding 3% Marvel (dried skimmed milk) solution at 250 µl per well. The plates were kept overnight at 4°C. Fish sera and PBS were added to the wells as before followed by mouse anti-tilapia monoclonal antibody (MAb). HRP anti mouse antibody diluted 1:1000 in PBS was added to wells. Development of assay and end point determination was as described earlier.

4.2.2.3. Western blot analysis

Sera from the fish of clonal line A and B fish post vaccination were screened by Western blot analysis. Sodium dodecyl sulphate polyacrylamide gel elcetrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970) using a 12% acrylamide separating gel. *A. hydrophila* T4 equivalent to 1 ml of a suspension with an optical density

(OD) of 1.0 at 610 nm were prepared in sample buffer and boiled for 5 min. After centrifuging at 9,300 g for 2 min, 200 µl of sample was added to a single large well, which was then subjected to electrophoresis for 45 min at 200 V. Bacterial antigens were transferred from the gel to sheets of nitrocellulose membrane by a wet blotting system using 50 V for 60 min. The gels were stained with 0.1 % (w/v) Coomassie blue R250, while the nitrocellulose membranes were washed with two changes of tris buffered saline with TTBS. Non-specific binding sites were blocked by incubating the membranes with 1.0 % (w/v) BSA in distilled water for 2 hours. They were washed twice in TTBS and placed in a Biorad multi-screen before applying the fish sera [1/10 dilution in phosphate buffered saline (PBS) (0.2M, pH 7.3)]. The nitrocellulose membranes were incubated overnight at 4°C, then were washed twice with TTBS. They were then incubated for 1 hour at 20°C, this time with mouse anti-tilapia monoclonal cell culture supernatant. They were washed twice with TTBS, then goat anti-mouse IgG-HRP conjugate (Diagnostic Scotland) (diluted 1/100 in TTBS) was applied to the membranes for 1 hour. Unbound conjugate was removed by washing the membranes twice with TTBS, followed by one wash with PBS. The assay was developed by incubating with chromogen [3-3-diaminobenzidine tetrahydrochlodide (DAB) (Sigma) (6mg)] dissolved in 10 ml of substrate buffer (20mM Tris hydrochloride, 500 mM sodium chloride, 30 µl hydrogen peroxide, pH 7.5) until bands appeared. The reaction was stopped by washing the membranes with distilled water for 10 min.

4.2.3. Standardisation of optimum dose of *A. hydrophila* T4 for challenge

A preliminary experiment was performed using URC fish, to optimise the dose of *A. hydrophila* T4 for the challenge. Bacterial suspensions were prepared at concentrations of $1x10^6$, $5x10^6$, $1x10^7$, $5x10^7$ and $1x10^8$ cells ml^{-1}. Viable plate counts were also done for each of the suspensions prepared and the exact concentrations used were 1.15×10^6, 5.2×10^6, 1.11×10^7, 4.9×10^7 and 0.9×10^8 cells ml^{-1} respectively. Eight fish were injected per bacterial concentration. Each fish received an i.p. injection of 0.2ml of one bacterial suspension. The results of the trial experiments are described in section 4.3.2.

4.2.3.1. Experimental challenge of fish with *A. hydrophila* T4

An experimental bacterial challenge was done to determine susceptibility of the vaccinated and non-vaccinated fish to *A. hydrophila*. The same bacterial strain was used in the challenge experiment.

The vaccinated and non-vaccinated fish were moved to the Aquatic Research Facility Unit of the Institute and were kept in a flow through system. The water temperature was maintained at 27°C ± 1°C. The fish were acclimatised for 7 days before being challenged on day 64 post-vaccination.

Bacteria were cultured in TSB for 24 hours at 22°C following removal from TSA slab. A bacterial suspension was made in 10 ml of sterile PBS and the concentration determined as described before in section 4.2.2.1.

All fish, vaccinated and non-vaccinated, were injected i.p. with 0.2ml of the prepared bacterial suspension of $1x10^7$ ml^{-1}, optimised at trial experiment. Fish were maintained for 14 days post-challenge. The fish were observed twice daily and checked for mortalities. Dead fish were removed from tanks, mortality recorded and bacterial swab

taken from the outer edge of skin lesions and from kidney and cultured onto TSA plates to re-isolate bacteria. Isolated bacteria was confirmed *A. hydrophila* using conventional methods (Barrow and Feltham, 1993) and the API 20E system (Bio Mericux, France).

4.2.4. Statistical analysis

The results serum antibody titre of different experimental groups of fish were analysed by multiple comparison analysis (Bonferroni multiple analysis) using a general linear model (Minitab V. 13). One-way ANOVA and 2x2 contingency analysis were also performed to test mortality rates after challenge with *A. hydrophila* T4.

4.3. Results

4.3.1. Immune response to *A. hydrophila* T4 in Nile tilapia experimental groups of fish

4.3.1.1. Optimisation of bacterial concentration for vaccine preparation

The results of the trial experiment to optimise the concentration of *A. hydrophila* T4 are shown in Fig. 4.1

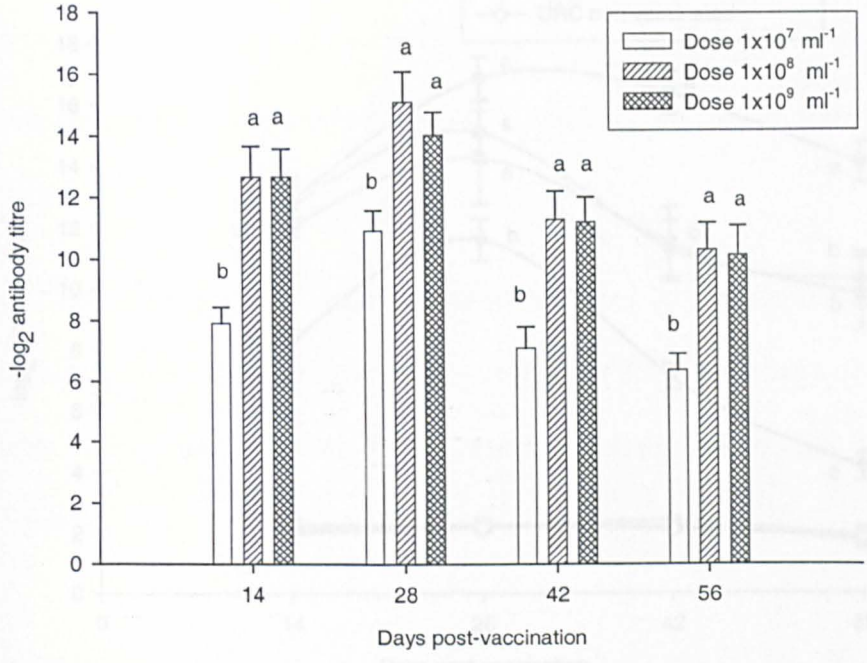


Fig. 4.1. Serum antibody titre of Nile tilapia (*Oreochromis niloticus*) after vaccination with different doses of heat-killed *A. hydrophila* T4. Data are presented as mean±SE of -log$_2$ antibody titre, (N=5). Lower case letters indicate significant differences (P<0.05).

The serum antibody titre increased significantly (P<0.05) with the increase in vaccine dose from 1x 10^7 ml^{-1} to 1x 10^8 ml^{-1} but differences in titre were not significant at any time point when the dose was increased from 1x10^8 ml^{-1} to 1x10^9 ml^{-1}. Therefore, 1x10^8 ml^{-1} was chosen as the *A. hydrophila* T4 concentration for vaccine preparation (Fig. 4.1).

4.3.1.2. Measurement of anti-*A. hydrophila* T4 serum antibody titre

All groups of experimental fish had a similar pattern of kinetics in the development of serum antibody titres after vaccination with *A. hydrophila* T4 but the magnitude of antibody titre measured differed between the different groups (Fig. 4.2).

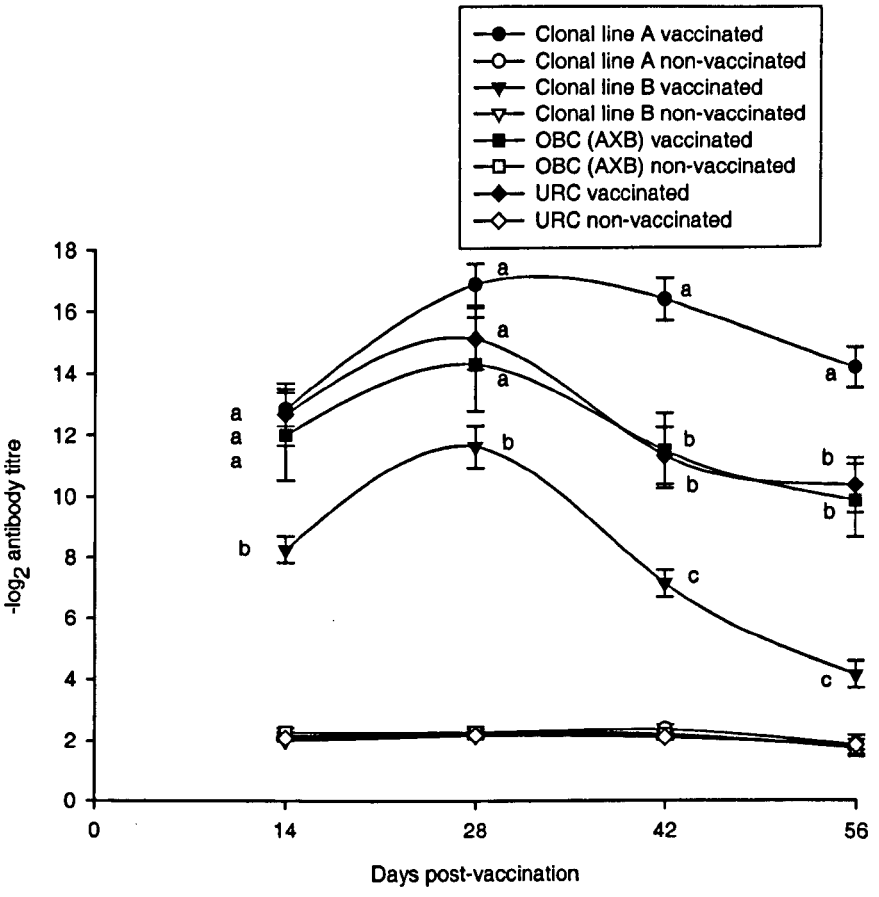


Fig. 4.2. Serum antibody titres in different experimental groups of Nile tilapia (*Oreochromis niloticus*) after vaccination with heat-killed *A. hydrophila* T4. Data are presented as mean±SE of $-\log_2$ antibody titre, (N=12). Lower case letters indicate significant differences ($P<0.05$) on same day.

All experimental groups of fish had the highest level of antibody titre on day 28 post-vaccination, after which the antibody titres declined in all experimental fish groups (Fig. 4.2).

The antibody titre of clonal line A fish was significantly ($P<0.05$) higher than the titre of clonal line B fish at all times over the course of the period of study. The OBC (AXB) and the URC fish both had lower antibody titres than that of clonal line A fish over the eight week post-vaccination period, but this difference was significant ($P<0.05$) only from day 42 post-vaccination. Clonal line B fish had a significantly ($P<0.05$) lower antibody titre than both OBC (AXB) and URC fish over the course of the experiment. The antibody titres of OBC (AXB) and URC fish were in between the values of antibody titres measured in clonal line A fish and clonal line B fish, but no significant difference was observed in the titres between OBC and URC fish at any time post-vaccination (Fig. 4.2).

Non-vaccinated fish from all groups had very low non-specific antibody titre but no difference was observed in this titre among the different groups of fish (Fig. 4.2). Mean anti-A. hydrophila titres of the experimental groups of fish are also presented in Appendix 4.

4.3.1.3. Western blot analysis

Differences were noted in the antigenic profiles of the sera between vaccinated clonal line A and clonal line B fish against *A. hydrophila* T4. A variety of bands were recognised in the profiles of *A. hydrophila* T4 in sera of clonal line A and clonal line B. The sera of clonal line A reacted with a major band around 35 kDa while sera from clonal line B reacted with several bands between 35 and 30 kDa. Sera from fish within each clonal line gave the same antigenic profile. The sere of URC recognised a different antigenic profile of *A. hydrophila* T4 (data not included).

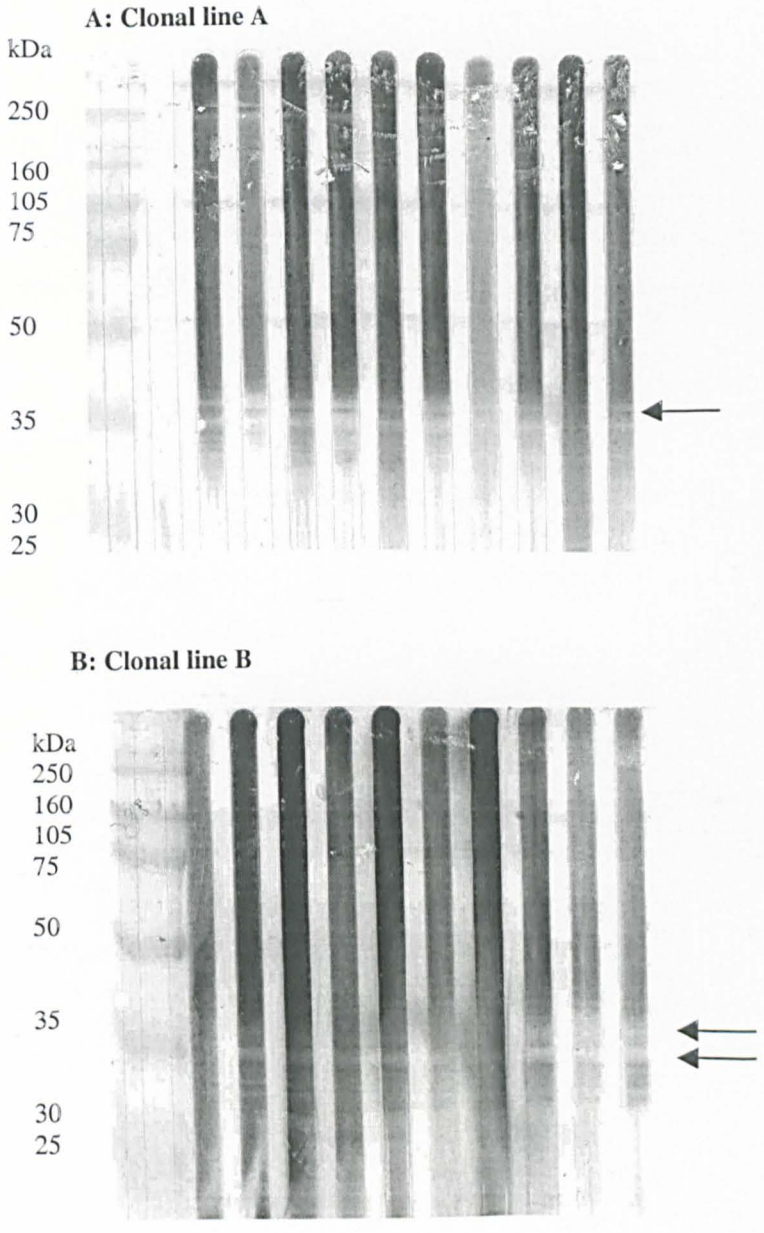


Fig. 4.3. Western blot analysis of sera from A: clonal line A, B: clonal line B following vaccinaton with heat killed *A. hydrophila* T4, 10 fish were analysed in each group. Left most lane is recombinant protein molecular weight marker (RPN 800).

4.3.2. Standardisation of optimum dose of *A. hydrophila* T4 for challenge

Fish started to die within 24 hours post-challenge. The infected fish showed slow movement and swam near the surface of water. Gross pathology of the infected fish showed haemorrhage on the body surface, base of fins and on the external side of the operculum. The anus was reddish, the abdomen swollen and the site of the injection was sometimes swollen and haemorrhagic.

Fish injected with different concentrations of *A. hydrophila* suspensions showed different mortality rates over the 10 day challenge period. All fish injected with $1x10^8$ cells ml^{-1} died within 24 hours post injection. At the end of the challenge period, 6, 4, 2 and 0 mortalities were recorded in tanks with fish injected with $5x10^7$, $1x10^7$, $5x10^6$ and $1x10^6$ cells ml^{-1} respectively. All fish were killed and samples taken to check level of infection in the surviving fish. Both the surviving fish injected with 5×10^7 cells ml^{-1} were heavily infected, 1 of the 3 surviving fish injected with 1×10^7 cells ml^{-1} was infected but the other 3 were not. None of the surviving fish injected with the two lowest concentrations was infected. It was assumed that a bacterial suspension with a concentration of $1x10^7$ cells ml^{-1} would be the ideal dose of bacteria to produce sufficient clinical signs but to keep the mortality at a minimum. Therefore $1x10^7$ cells ml^{-1} was selected as the standard dose for the bacterial challenge experiment.

4.3.2.1. Experimental challenge with *A. hydrophila* T4

Figs 4.4, 4.5 and Table 4.1 show the results of experimental challenge of vaccinated and non-vaccinated fish with *A. hydrophila* T4. In the vaccinated group, clonal line B had significantly ($P<0.05$) higher mortality than clonal line A, OBC (AXB) and URC fish. OBC (AXB) fish also had significantly ($P<0.05$) higher mortality than clonal line A. No significant differences in mortality were observed between clonal line A fish and URC fish or between OBC fish and URC fish (Fig. 4.4 A).

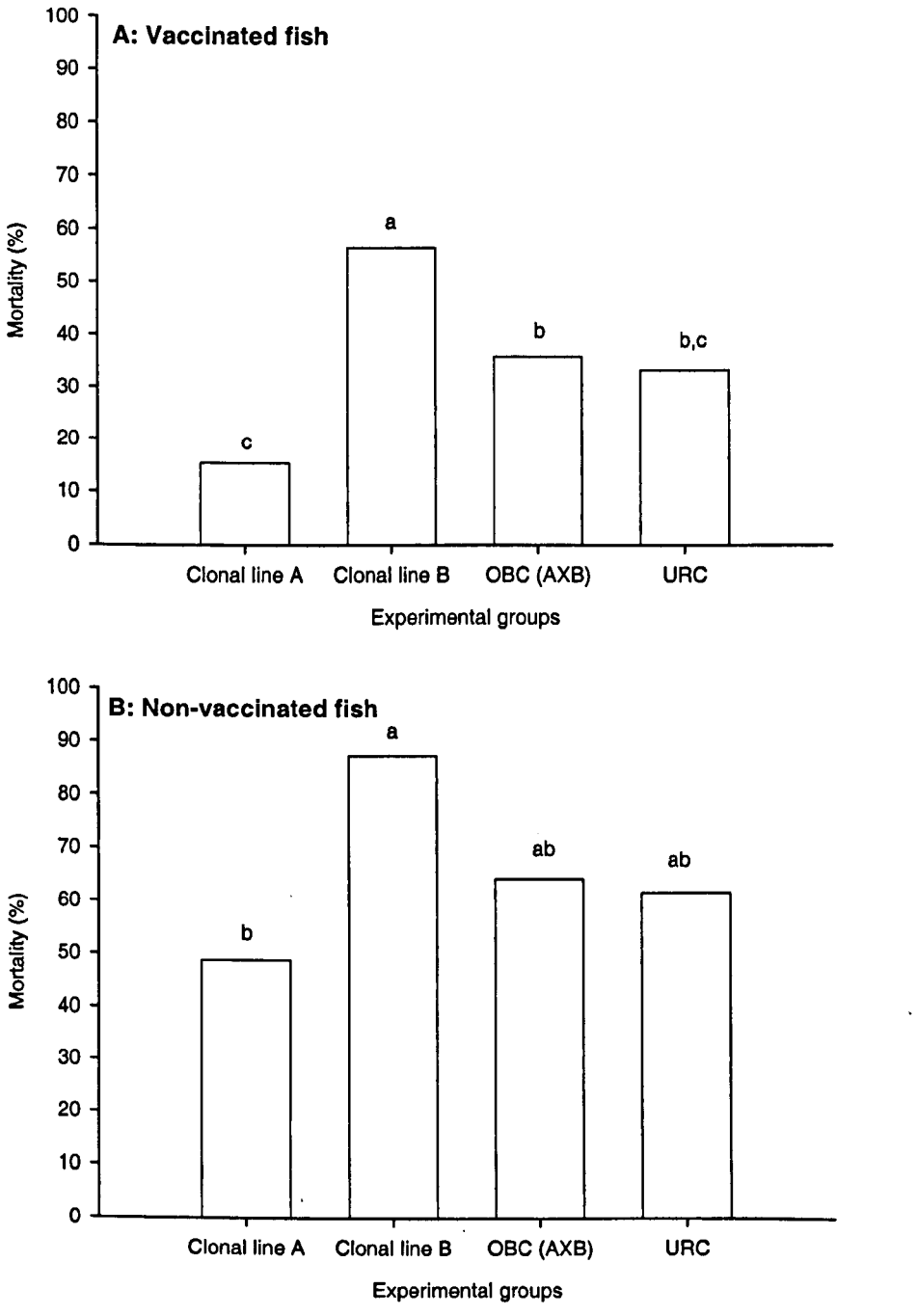


Fig. 4.4. The percentage of mortality in different experimental groups of Nile tilapia after challenge with *A. hydrophila* T4. A:Vaccinated fish, B:Non-vaccinated fish. Different lower case letters indicate significant ($P<0.05$) differences.

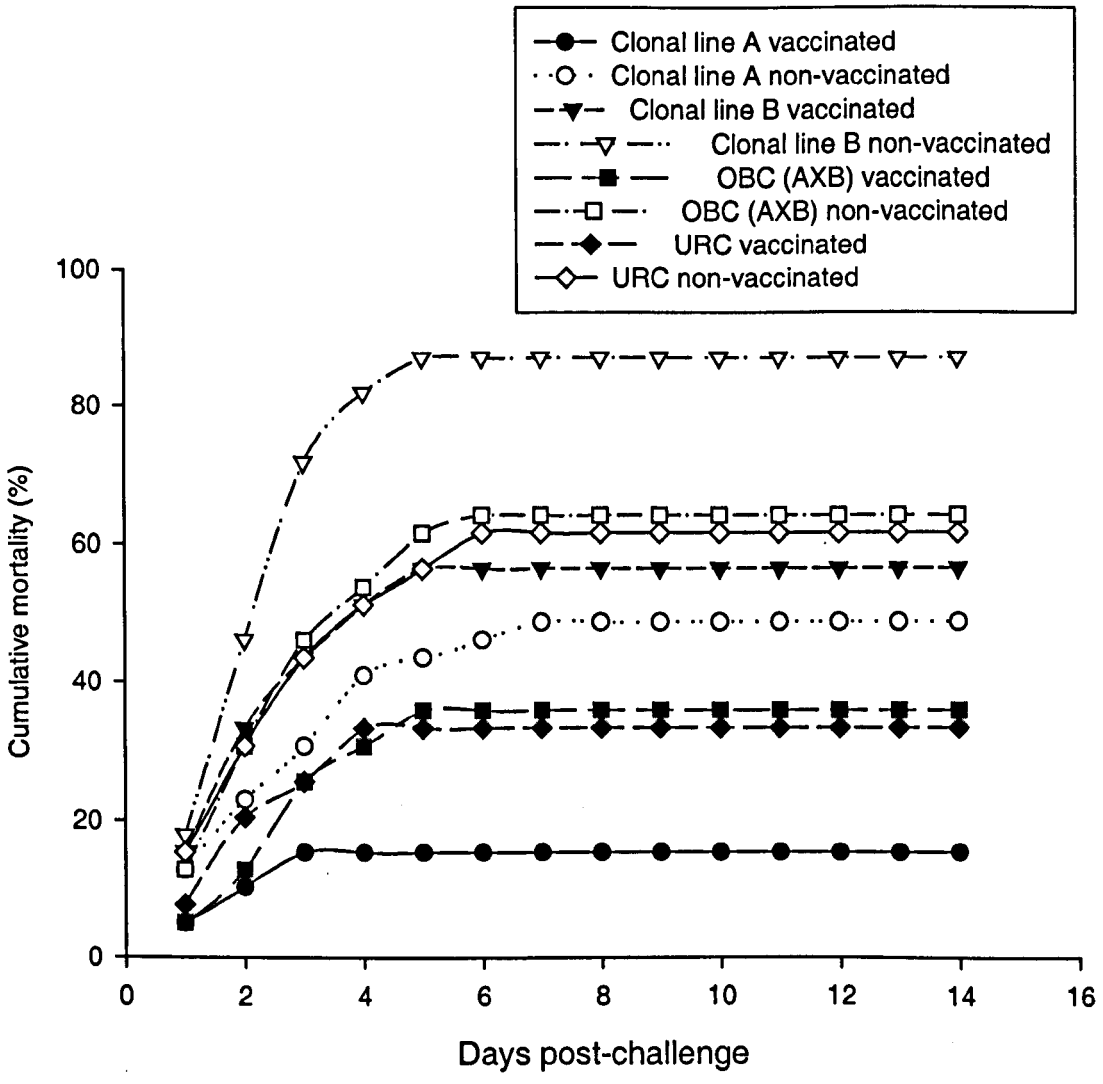


Fig. 4.5. Cumulative mortalities in experimental groups of Nile tilapia (*Oreochromis niloticus*) during an artificial challenge with *A. hydrophila* T4. (N=39).

In non-vaccinated fish, clonal line B fish had significantly ($P<0.05$) higher levels of mortality than clonal line A fish but no significant difference in mortality was found between clonal line A and OBC and URC fish and between clonal line B, OBC (AXB) and URC fish (Fig. 4.4 B). It was observed that vaccinated clonal line B had a higher mortality than non-vaccinated clonal line A (Fig.4.5).

Table 4.1. 2X2 contingency table of mortality in different experimental groups after challenge with *A. hydrophila* T4 in vaccinated and non-vaccinated group. * significant at P<0.05; RLP**: Relative Level of Protection.

Groups	Treatments	Dead				Alive	% mortality	χ^{2*}	RLP**
		Tank 1	Tank 2	Tank	Total				
Clonal line A	Vaccinated	3	2	1	6	33	15.4	9.9	68.4
	Non-vaccinated	6	5	8	19	20	48.7	DF = 1, P-Value = 0.002	
Clonal line B	Vaccinated	8	6	8	22	17	56.4	9.1	35.3
	Non-vaccinated	13	13	8	34	5	87.2	DF = 1, P-Value = 0.003	
OBC (AXB)	Vaccinated	4	6	4	14	25	35.9	6.2	44.0
	Non-vaccinated	8	8	9	25	14	64.1	DF = 1, P-Value = 0.013	
URC	Vaccinated	5	4	4	13	26	33.3	6.2	45.8
	Non-vaccinated	9	7	8	24	15	61.5	DF = 1, P-Value = 0.013	

$$RLP^{**} = 100 - \frac{\text{\% mortality in vaccinated group}}{\text{\% mortality in control group}} (100)$$ (Newman and Majnarich, 1982)

There was a significant difference in mortality between vaccinated and non-vaccinated fish in all experimental groups of fish (Table 4.1).

4.4. Discussion

The aim of this study was to compare the specific immune responses of two Nile tilapia inbred clonal lines (clonal line A and B), one outbred clonal line (OBC: AXB) and unrelated control group (URC) following vaccination with *A. hydrophila* T4 and to compare disease resistance between the experimental groups of fish after an experimental challenge with the same pathogen.

In the present study, clonal line A fish showed a significantly higher serum antibody titre than all other three groups of fish and clonal line B fish showed a significantly lower titre than the other three groups of fish after vaccination against *A. hydrophila* T4. Antibody titres of OBC (AXB) and URC fish were in between those of clonal line A and clonal line B and significantly different from both clonal lines from day 42 post-vaccination but were not significantly different from each other. Comparison of the antigen profile of *A. hydrophila* T4 recognised by the sera of high responding clonal line A and low responding clonal line B by Western blot analysis revealed differences between the two clonal lines. This might be due to differences in antigen processing and presentation between the two clonal lines. Fish within each clonal line had the same antigenic profile.

Experimental challenge as a means of studying disease resistance in fish is well documented in the literature (Van Muiswinkel *et al.*, 1990; Withler and Evelyn, 1990; Gjedrem *et al.*, 1991; Wiegertjes *et al*, 1993a, 1995a,b; Gjoen *et al.*, 1994; Stromsheim *et al.*, 1994a,b; Gjedrem and Gjoen, 1995). The experimental challenge in the vaccinated group resulted in clonal line A having a significantly lower level of mortality than clonal line B and OBC (AXB) fish while clonal line B fish had significantly higher

mortality than clonal line A, OBC (AXB) and URC fish. OBC (AXB) fish mortality was in between, significantly higher than clonal line A but significantly lower than clonal line B. Mortalities in clonal line A and URC were not significantly different. In the non-vaccinated group, clonal line B had significantly higher mortality than clonal line A. OBC (AXB) and URC had mortalities in between clonal line A and B but not significantly different from either group. These results showed that there was an inverse relationship between antibody production and susceptibility to disease. Clonal line B was highly susceptible to *A. hydrophila* T4 infection and protection against *A. hydrophila* T4 infection after vaccination was low in that clonal line compared to the vaccinated fish of other experimental groups as well as non-vaccinated fish of clonal line A. The mortality in all experimental groups of fish reached a plateau at day 6 post-challenge. Although serum antibody titre was not measured on the first six days post-vaccination or post-challenge, it was assumed that clonal line A had significantly higher serum antibody titre than clonal line B and that was responsible for significantly lower mortality in clonal line A fish than clonal line B fish.

Sarder *et al.* (2001) used the same clonal lines A and B and OBC (AXB) to compare resistance to *A. hydrophila* T4 without prior vaccination. Clonal lines A and B and OBC (AXB) had a cumulative mortality of 3.3%, 56%, and 20% over the 6 day experimental challenge period showing almost identical relationship to the present experiment.

The relative level of protection (RLP) was not absolute in any groups. Ruangpan *et al.* (1986) in a study on Nile tilapia vaccination with formalin killed *A. hydrophila* with or without Freund's complete adjuvant, reported absolute RLP when vaccinated fish were challenged two, three and five weeks post-vaccination. Formalin killed vaccine

might have better efficacy than heat killed vaccine in rendering protection against *A. hydrophila* in Nile tilapia. A commercial vaccine for this pathogen has still not been introduced (Schnick *et al.*, 1997). Rahman and Kawai (2000) reported that outer membrane proteins (OMP) of *A. hydrophila* have protective immunogenicity in goldfish and it might be useful to develop vaccines by selecting OMP as antigens. Effectiveness of a vaccine should not be measured in terms of protection alone but also by its ability to elicit a specific immune response in fish to protective antigens (Hastings, 1988). Antibody titre has been measured following vaccination but often did not correlate with protection for *A. salmonicida* (McCarthy *et al.*, 1983; Olivier *et al.*, 1985). However, some investigations have shown antibody titres to protective antigens such as *A. salmonicida*, to be correlated with protection (Hirst and Ellis, 1994; Lund *et al.*, 1995a,b; Ellis, 1997), as observed in the present study for *A. hydrophila* T4.

Studies on antibody response against test antigens have shown both positive and negative correlation with disease resistance in fish and other animals. In inbred mouse lines selected for high and low response to SRBC (Biozzi *et al.*, 1979), stronger antibody response in the high lines caused an increased resistance to extracellular pathogens, and the high responder mice were more susceptible to infection with typically intracellular pathogens such as *Salmonella typhimurium* (Biozzi *et al.*, 1984). Van der Zijpp and Nieuwland (1986) also divergently selected high and low responding inbred chicken lines for antibody response against SRBC. Scott *et al.* (1994) reported that high and low responding poultry lines exhibited high and low responses against *Brucella abortus* even though they were selected for antibody responsiveness to SRBC. The antigenic component of *B. abortus* is classified as a Type-1 T-independent antigen which

stimulates B cells with little assistance from T helper cell but does require macrophage like accessory cells to induce antibody formation (Mosier and Subbarao, 1982). Common carp were divergently selected for high or low antibody response to DNP-KLH and inbred lines were produced by gynogenesis (Wiegertjes *et al.*, 1994). The divergently selected carp lines were infected with *Trypanoplasma borreli*, a haemoflagellate parasite of carp. For this particular disease model, an apparent relationship between susceptibility and lack of antibody formation has been suggested. Susceptibility to the parasite was dependent upon the immune response type of the carp lines, i.e. the high-responder carp line was more resistant than low-responder carp line (Wiegertjes *et al.*, 1995a). It was suggested that the high and low responder homozygous fish lines differed for at least one gene with a major influence to *T. borreli* (Wiegertjes *et al.*, 1996b). On the other hand, antibody response to diphtheria toxoid correlated negatively with resistance to *Vibrio anguillarum* in rainbow trout and Atlantic salmon (Eide *et al.*, 1994). In the present study, the experimental groups of Nile tilapia had similar antibody responses to the test antigens as well as to the disease causing pathogen. Antibody production also reflects resistance to *A. hydrophila* infection. Therefore antibody response to test antigens can be used as a possible indicator of disease resistance to *A. hydrophila* in these experimental groups of Nile tilapia.

Non-specific immune and physiological parameters have also been studied for possible correlation with disease resistance. Brown trout selected for high mucus precipitin level also had higher resistance to *A. salmonicida* infection (Cipriano and Heartwell, 1986). In rainbow trout, a high stress response group had high cortisol level and lysozyme activity but low serum haemolytic activity and had higher mortality after

challenge with *A. salmonicida* than low responding group. This high stress responding group had lower mortality than a low responding group after *V. anguillarum* challenge (Fevolden *et al.*, 1992). Nile tilapia and Coho salmon strains with higher response regarding phagocyte respiratory burst activity, plasma lysozyme activity and higher differential leucocyte counts before and during initial infection with *V. parahaemolyticus* and *V. anguillarum* respectively, also had higher survival following challenge (Balfry *et al.*, 1994). Lysoyme activity in stressed fish had a negative correlation with survival against *A. salmonicida* (causing furunculosis), *Renibacterium salmoninarum* (causing bacterial kidney disease) and *A. salmonicida* (causing cold water vibriosis) in Atlantic salmon but cortisol level of stressed fish had no correlation (Fevolden *et al.*, 1994). Atlantic salmon families with high levels of serum iron was more susceptible to *V. anguillarum* infection (Ravandal *et al.*, 1994). Hollebecq *et al.* (1995) reported that resistance of rainbow trout to *A. salmonicida* could be correlated with serum bactericidal activity but had no clear relation with complement spontaneous haemolytic activity.

Biozzi *et al.* (1984) reported high rate of macrophage metabolism in the low responder inbred mouse line and suggested an inverse relationship between genetic regulation of macrophage activity and antibody responsiveness. On the other hand, chickens selected for high and low antibody response (Van der Zijpp and Nieuwland, 1986) did not differed in respect to phagocytic activity (Kreukniet *et al.*, 1994) and macrophage ability to respond to antigenic challenge (reviewed by Scott *et al.*, 1994).

The non-specific immune response of the same two clonal lines of Nile tilapia (clonal line A and B) used in the present study differed significantly regarding the percentage of

macrophages containing phagocytosed bacteria but did not differ significantly in the number of macrophages per gram of kidney tissue and in their lysozyme activity, after an experimental challenge with *A. hydrophila* T4 (Sarder *et al.*, 2001). Lysozyme activity after challenge had shown positive and negetive correlation with survival after challenge (Fevolden *et al.*, 1992, 1994). In this study no correlation could be established with survival after challenge and serum lysozyme activity reported by Sarder *et al.* (2001). Though the numbers of macrophages were not different between the two clonal lines, the level of their activity was different resulting in a significant difference in the percentage of macrophages with phagocytosed bacteria. This might lead to differences in antibody processing and presentation between the two clonal lines.

Kincaid (1993) reported that quantitative traits associated with reproduction or physiological efficiency such as growth and survival are often affected by inbreeding depression, which in turn might improve in hybrid lines. Superior viability and disease resistance in hybrids have also been reported by Bakos (1987) and Ilyasov (1987). In the present study, in all experiments, OBC (AXB) fish had intermediate response and resistance, significantly lower response and resistance than clonal line A and significantly higher response and resistance than clonal line B. This indicated additive parental effects on OBC (AXB) fish. Sarder (1998) reported that the OBC (AXB) fish showed higher level of lysozyme (though not significant) from both the parents, a significantly higher phagocytic activity than the low responding parent (clonal line B) and the number of macrophages per gram of kidney tissue was in between the two parents and suggested a likely positive heterosis in the group of fish. Hines *et al.* (1974) in their study on different common carp strains found that hybrids from the cross

between high and low responder line were relatively resistant, indicating dominant inheritance of the resistant genotype. F1 crosses between inbred lines resulted in progeny unaffected by infection although both or one of the parents were highly susceptible.

From the above discussion it is revealed that clonal line A had significantly higher specific immune responses than clonal line B fish. Clonal line A also had significantly higher resistance to *A. hydrophila* T4 infection than clonal line B fish. Any difference in specific immune response and resistance to disease in the two clonal lines should be due to genetic difference between the two fully inbred clonal lines of Nile tilapia. Though specific immune response and disease resistance to *A. hydrophila* infection was higher in clonal line A than the URC fish, the differences were not significant. Specific immune response and disease resistance of OBC (AXB) fish were in between the response and disease resistance of the two clonal lines, and were significantly higher than clonal line B fish. Although disease resistance was significantly increased in OBC (AXB) fish to that of the more susceptible parent (clonal line B), the more resistant parent (clonal line A) has high potential to be used as a valuable tool to produce subsequent generations. Individual high responders from subsequent generations can be identified and selected for the establishment of the disease resistant population of fish. Clonal line A can also be used to study the genetic variation for the traits responsible for this resistance within the Stirling population and therefore could be used in quantitative trait loci (QTL) analysis.

CHAPTER 5

STUDY ON THE MITOGEN STIMULATION OF LYMPHOCYTES IN CLONAL LINES OF NILE TILAPIA *Oreochromis niloticus*

5.1. Introduction

The importance of haematology and immunology in the health assessment of higher vertebrates has led to the application of a variety of haematological and immunological techniques for the assessment of fish health (Hesser, 1960; Blaxhall, 1972; Hickey, 1976; Wedemeyer and Yasutake, 1977). Techniques adopted have classically been descriptive in nature and include techniques such as haematocrit (Soivio and Oikari, 1976; Munkitrick and Leatherland, 1983), haemoglobin (Sniesko, 1960), number of leukocytes or leucocrit (McLeay and Gordon, 1977; Wedemeyer *et al.*, 1983), chromosomal aberrations (Al-Sabti, 1983), macrophage aggregates (Wolke *et al.*, 1985) or combinations of these parameters.

Cellular immune function is an important component of both cell mediated and humoral immunity and is crucial in assessing immunocompetence of an organism (Tillitt *et al.*, 1988). Lymphocyte proliferation or stimulation assays, using mitogens, is commonly known as lymphocyte activation (LA) and is an extremely useful test to assess cellular immune function. A variety of functional studies including LA technique have been used in mammalian toxicology to assess the immunological functions of cell mediated immunity and these have been recommended for routine screening to assess the immune status of the animal (Vos and Moore, 1974; Vos, 1977, 1981; Koller, 1979; Sharma, 1981; Bleavins and Aulerich, 1983; Bleavins *et al.*, 1983; Greenlee *et al.* 1985, Swart *et al.*, 1994). LA *in vitro* is also used in human medicine to assess cellular immunity in such cases as immunodeficiency, autoimmunity, infectious diseases and cancer (Stites *et al.*, 1982). Reports on the utilisation of functional immunological tests for the evaluation of fish health are scarce in the literature covering the area of aquatic

toxicology and fish disease (Zeeman and Brindley, 1981). Recently, the effects of drugs and other toxic substances, stress hormones, immunostimulants used in feed and antigens on immunological function in fish have been assessed by measuring lymphocyte proliferation by mitogens. Both enhanced and reduced lymphocyte proliferation have been reported in different studies (Grondel and Boestan, 1982; Grimm, 1985; Grondel *et al.*, 1985; Dunier *et al.*, 1991; Dannevig *et al.*, 1993; van der Heidjen *et al.*, 1995; El-Gendy *et al.*, 1998; Tachibana *et al.*, 1997; Verlhach *et al.*, 1998). Studies on mitogen induced proliferation of lymphocytes isolated from anterior kidney and spleen of diseased Atlantic menhaden *Brevoortia tyranus* has been reported by Faisal and Hargis (1992). McKinney and Schmale (1993) used LA by mitogens to study immunocompetence and disease susceptibility of juvenile damselfish *Pomacentrus partitus* to neurofibromatosis related tumor formation.

Mitogens are agents capable of inducing cell division in T or B cells. Unlike immunogens, which only activated lymphocytes bearing receptors specific for that immunogen, a mitogen can activate many clones of B or T cells irrespective of their antigen specificity and because of this ability, mitogens are known as polyclonal activators (Kuby, 1997).

A variety of different agents function as mitogens. Several common mitogens are sugar binding proteins called lectins, which specifically bind to different glycoproteins on the surface of various cells, including lymphocytes. Binding of lectin molecules to membrane glycoproteins can lead to agglutination or clustering of the cells which in turn may trigger cellular activation and proliferation. Some mitogens preferentially activate B cells, some preferentially activate T cells and some activate both populations. Three

common lectins of mitogenic activity are Concanavalin A (Con A), phytohemagglutinin (PHA) and pokeweed mitogen (PWM). Each of these mitogens bind to different carbohydrate residues on the glycoprotein (Goldstein and Hayes, 1978).

Not all mitogens are lectins. The lipopolysaccharide (LPS) component of the cell wall of Gram negative bacteria functions as a B cell mitogen. The mitogenic activity is due its lipid moiety, which is thought to interact with its plasma membrane, resulting in a cellular activation signal (Andersson *et al.*, 1972).

The majority of the evidence supporting the presence of T and B lymphocytes populations in higher vertebrates have come from various functional assays performed *in vitro*. One of the functional assays most widely used *in vitro* to study fish lymphocyte heterogeneity is the proliferative responses of different lymphocyte populations to mitogens. A mitogenic response to the lectins Con A and PHA is specific for T lymphocytes in mammals and birds while LPS is considered a B lymphocyte mitogen in these species (Weber, 1973).

Studies with a number of different fish species have revealed that their lymphocytes respond to mitogens resulting in LA (Etlinger *et al.*, 1976; Cuchens and Clem, 1977; Sigel *et al.*, 1978; Al-Sabti *et al.*, 1983; Blaxhall, 1983; Faulmann *et al.*, 1983; Warr and Simon, 1983; Caspi *et al.*, 1984). Lymphocytes from teleosts, although not as clearly defined as those from higher vertebrates, appear to have discernible populations of T and B lymphocytes (Clem *et al.*, 1981; Warr and Simon, 1983). Evidence to support this fact comes from differential response of cells obtained from different lymphoid tissues (spleen, thymus and anterior kidney) and peripheral blood of fish to the same mitogens, used to stimulate proliferation of lymphocytes of higher vertebrates, i.e. classical T cell

mitogens, PHA and Con A and B cell mitogens, LPS and purified protein derivatives (PPD). Most of the studies on mitogen responses on teleost fish have been done to determine lymphocyte heterogeneity in different lymphoid organs of rainbow trout (Etlinger *et al.*, 1976; Warr and Simon, 1983; Tillitt *et al.*, 1988; Reitan and Thuvander, 1991; Kehrer *et al.*, 1998), bluegill *Lepomis macrochirus* (Cuchens and Clem, 1977), channel catfish (Clem *et al.*, 1984), common carp (Caspi *et al.*, 1984); holostean fish spotted gar *Lepisosteus platyrhinchus* (Luft *et al.*, 1994) and European seabass *Dicentrarchus labrax* (Galeotti *et al.*, 1999) or to optimise lymphocyte culture conditions in channel catfish (Faulmann *et al*, 1983; Sizemore *et al.*, 1984); common carp (Rosenberg-Wiser and Avtalion, 1982; Troutaud *et al.*, 1995), rainbow trout, coho salmon and chinook salmon (DeKoning and Kaattari, 1991, 1992) rather than to assess the immune response in toxicological studies (Tillitt *et al.*, 1988). Mitogens have also been used to enhance cell division in cell cultures used for fish karyotyping from metaphase chromosome spreads (Fenocchio and Bertollo, 1988).

Establishing a lymphocyte mitogen assay for fish lymphocytes would be potentially useful for assessing the health status of fish and the status of their immune system during immunotoxicological screening tests, in fish culture systems and in wild populations (Tillitt *et al.*, 1988).

5.1.1. The aim of the study

The aim of the present study was to determine differences in the polyclonal activation of B and T lymphocyte populations in the lymphoid organs between experimental groups of Nile tilapia clonal lines (clonal lines A and B), an outbred clonal line (OBC AXB) and unrelated control (URC) fish and to use LA to compare the immune response of the experimental groups of fish.

5.2. Materials and methods

5.2.1. Fish stocks and maintenance

Fish used in this study came from the Tilapia reference collection at the Institute of Aquaculture, University of Stirling. Production, propagation and general husbandry of the fish used in the study has previously been described in Chapters 2 and 3.

Twenty fish from each of clonal line A and (OBC AXB) and 16 fish from each of clonal line B and URC were used for this assay.

5.2.2. Isolation of lymphocytes

Lymphocytes were isolated from peripheral blood, spleen and head kidney using the same method as described earlier in Section 3.2.2.1. Isolated lymphocytes were adjusted to a concentration of $5x10^6$ ml^{-1} with L-15 Leibovitz (Sigma) containing 5% heat inactivated foetal bovine serum (FBS, Sigma) and supplemented with penicillin/streptomycin (100 units of penicillin and 50 µg of streptomycin ml^{-1} of cell culture medium, Sigma), L-glutamine (2mM, Sigma) and sodium pyruvate (1% v/v, Sigma).

5.2.3. Mitogen stimulation assay

Mitogen stimulation assay was performed following the protocol described by Findlay (1994), which was modified from the methods of Tillitt *et al.* (1988) and Reitan and Thuvander (1991).

Con A (Concanavalin A from *Canavalia ensiformis*, Sigma) was used as a T lymphocyte mitogen and Lipopolysaccharides (LPS from *Escherichia coli* 055:B5, Sigma) was used as a B cell mitogen.

The mitogens were reconstituted and diluted to the concentrations of 40 μg ml^{-1} for Con A and 200 μg ml^{-1} for LPS in L-15 Leibovitz/ 5% FBS with supplements. Lymphocyte cell suspensions from blood, spleen and head kidney were added at 100 μl per well to duplicate wells of 96 well round bottomed plate (NUNC ™, Denmark) followed by 100 μl of either Con A or LPS giving a final volume of 200 μl per well, while 100 μl of PBS was added to the control wells. The plates were incubated in an airtight and sterile moist chamber at 27°C for 72 hours after which 1.0 μCi $well^{-1}$ of ^{3}H-thymidine was added and the plate was incubated for a further 24 hours. After this time, the contents of each well were harvested onto filter paper discs (Skatron Instruments, UK) using a Titertex cell harvester (Flow Laboratories, UK) and prepared for scintillation counting. Only the ^{3}H-thymidine that has been incorporated into T or B cells was retained by the filter paper discs. The filter paper discs were air-dried and each disc was placed in 6 ml scintillation vial (Packard BioScience). In each vial, 4 ml scintillation fluid (Ultima Gold ™, Packard BioScience) was added and vortexed vigorously. The rate of ^{3}H-Thymidine uptake was assayed using a 1900 liquid scintillation analyser (Canberra-Packard, Tricarb). The degree of stimulation for each mitogen was calculated using a stimulation index (S.I.)

$$\text{S.I.} = \frac{\text{mean counts per minute for simulated culture}}{\text{mean counts per minute for unstimulated culture}}$$

5.2.4. Statistical analysis

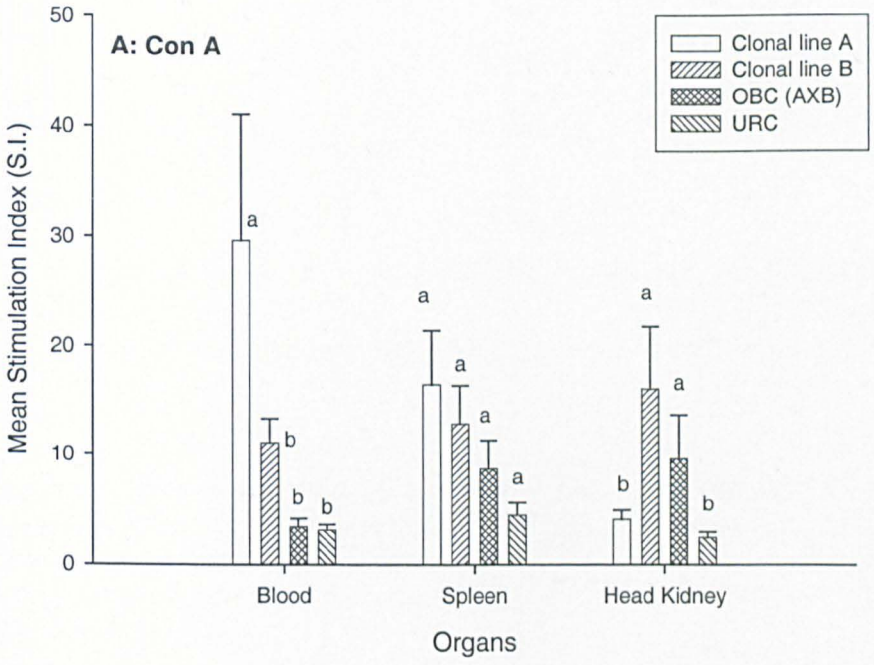
The data was analysed by ANOVA (Bonferroni multiple analysis) using general linear model (Minitab V. 13).

5.3. Results

Stimulation indices of lymphocytes from the same organs of different groups of experimental fish when incubated with either Con A or LPS are presented in Fig. 5.1.

Clonal line A had significantly ($P<0.05$) higher S.I. than the other groups of fish in peripheral blood lymphocytes stimulated with Con A. Clonal line B had significantly ($P<0.05$) higher S.I. than clonal line A and URC in head kidney lymphocytes stimulated with Con A.

Clonal line A had significantly ($P<0.05$) higher S.I. than URC in peripheral blood lymphocytes stimulated with LPS. Clonal line B had significantly ($P<0.05$) higher S.I. than OBC (AXB) and URC in spleen lymphocytes stimulated with LPS.



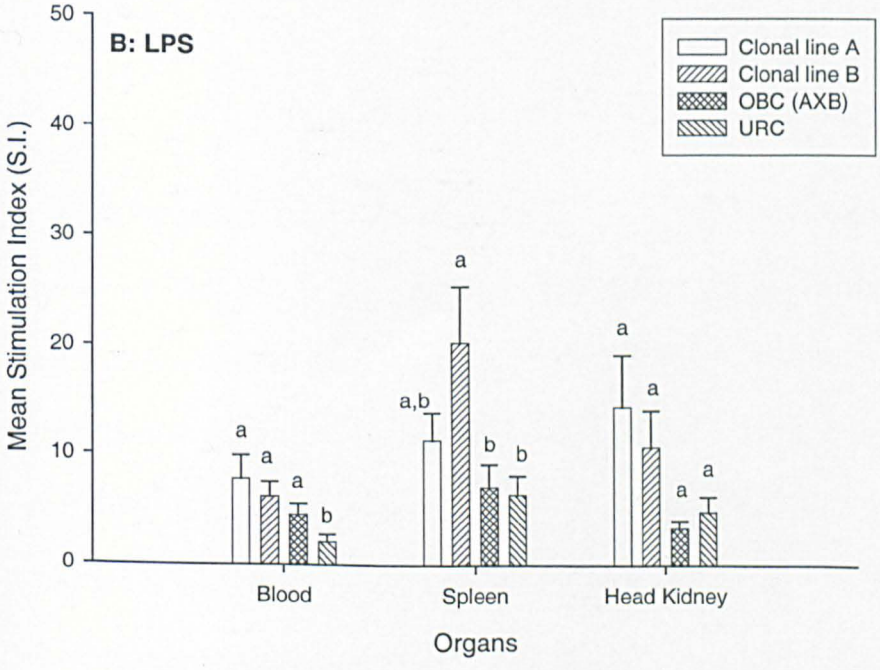


Fig. 5.1. Stimulation indices of lymphocytes from different groups of experimental Nile tilapia *Oreochromis niloticus*. Data presented as mean S.I. ± SE. Different letters indicate significant differences ($P<0.05$) between means of different groups of experimental fish for the same organ. A: Con A; B: LPS.

The CPM of stimulated lymphocytes by mitogens (Con A and LPS) and non-stimulated lymphocytes of the experimental groups of fish are also presented in Appendix 5.

A comparison of the S. I. obtained with of lymphocytes from the same tissue within same group of experimental fish when stimulated with Con A and LPS is shown in Table 5.1.

Table 5.1. Stimulation indices of lymphocytes from different tissues of experimental groups of Nile tilapia *Oreochromis niloticus*. Data presented as mean S.I. ± SE. Different lower case letters indicate significant (P<0.05) differences between means of S.I. of lymphocytes from the same tissue in the same group of fish, when stimulated with Con A or LPS.

Experimental group	Tissues					
	Blood		Spleen		Head kidney	
	Con A	LPS	Con A	LPS	Con A	LPS
Clonal line A	29.6 ± 11.4^{a}	7.8 ± 2.1^{b}	16.5 ± 4.9^{a}	11.3 ± 2.4^{a}	4.0 ± 0.8^{b}	14.3 ± 4.8^{a}
Clonal line B	11.1 ± 2.2^{a}	6.1 ± 1.3^{a}	12.8 ± 3.5^{a}	20.2 ± 5.1^{a}	16.1 ± 5.7^{a}	10.7 ± 3.3^{a}
OBC (AXB)	3.4 ± 0.7^{a}	4.5 ± 0.9^{a}	8.8 ± 2.5^{a}	7.0 ± 2.0^{a}	9.7 ± 3.9^{a}	3.3 ± 0.6^{a}
URC	3.1 ± 0.4^{a}	2.0 ± 0.6^{a}	4.4 ± 1.1^{a}	6.3 ± 1.7^{a}	2.4 ± 0.5^{a}	4.7 ± 1.3^{a}

Lymphocytes from peripheral blood of clonal line A fish had significantly (P<0.05) higher S.I. with Con A than with LPS. Head kidney lymphocytes of this clonal line fish had significantly (P<0.05) higher S.I. when stimulated with LPS compared to when stimulated with Con A. In clonal line B fish, the S.I. with Con A was higher than seen with LPS in lymphocytes from peripheral blood and from head kidney but spleen lymphocytes appeared to be better stimulated with LPS than with Con A. However, no significant differences were observed (Table 5.1).

The lymphocytes from the spleen and head kidney of OBC (AXB) fish had a higher S. I. with Con A than seen with LPS while and peripheral blood lymphocytes gave a better stimulation with LPS than with Con A but there were no significant difference. In

URC fish, the S.I. of spleen and head kidney lymphocytes with LPS were higher than with Con A and the S.I. of peripheral blood lymphocytes were higher with Con A than was seen with LPS, but no significant differences were noted (Table 5.1).

5.4. Discussion

The effects of drugs and other toxic substances and antigens on the immunological mechanism of fish are routinely measured by lymphocyte proliferation using mitogens and both enhancement and reduction in lymphocyte proliferation have been reported (Grondel and Boestan, 1982; Grondel *et al.*, 1985; Dunier *et al.*, 1991; Dannevig *et al.*, 1993; Van der Heidjen *et al.*, 1995; El-Gendy *et al.*, 1998). Immunological tests, like the lymphocyte proliferation assay, give information about a particular immunological process occurring at a fixed time during an immune response. While measurement of blastogenesis gives an indication of the ability of the lymphocytes to respond and proliferate to an activation signal which is essential for an adequate immune response, this polyclonal activation of lymphocytes provide little information about the efficacy of the defence system as a whole such as the ability of the cells to develop into effector cells. Immunological function may be more or less amplified or compensated by possible interaction with different immune components i.e. antigen processing and presentation and non-specific defence systems (Reitan and Thuvander, 1991; Van der Heidjen *et al.*, 1995). Lymphocyte stimulation with mitogens is widely used to study general immunocompetence (Urbaniak *et al.*, 1986) but stimulation of lymphocytes by antigens can be used to determine whether an animal has been sensitised to a given antigen by measuring lymphocyte proliferation and/or production of specific antibodies (Ljungman *et al.*, 1985). Blastogenic transformation induced by an antigen expresses a specific response while blastogenic transformation evoked by lectins or LPS is a more general response of large segments of lymphocytes regardless of specificity, commitment or immune status of the animal (Siegel *et al.*, 1978). Mitogenic activity of

lymphocytes is not linked to antigen specific stimulation of antibody production (Kaattari and Yui, 1987).

The specific immune response to antigens observed with these experimental groups of fish (Chapter 3 and 4) was different from the response obtained with the mitogens as observed in this chapter. The fish were not exposed to any toxic substances, drugs or antigens prior to mitogen assay for this experiment. This result indicates that there might not be any significant differences in the total lymphocyte populations (both T and B cells) among the groups of experimental fish but expansion levels of specific B lymphocyte population (i.e. specific for a particular antigen after exposure) may differ between the groups. Attempts were made to separate T and B lymphocyte populations from different lymphoid tissues of the experimental groups of fish using mouse anti-tilapia Mab produced against the heavy chain and light chain cells of Nile tilapia IgM on cytocentrifuged preparations of cell suspensions. But results were inconclusive.

Grimm (1985) reported on an association between the stress induced hormone cortisol and suppression of mitogen induced proliferation of lymphocytes from peripheral blood of plaice. Outbreaks of disease in fish are frequently associated with environmental stressors, which result in secretion of cortisol. In Atlantic menhaden affected with ulcer disease syndrome, lymphocytes isolated from spleen and head kidney of severely affected and recovering fish had higher proliferation when stimulated with a number of different mitogens than lymphocytes from disease free control fish. This significant augmentation of proliferation response to mitogens in diseased fish indicated a higher polyclonal activation of lymphocytes in fish suffering from disease (Faisal and Hargis, 1992). Head kidney lymphocytes from Atlantic salmon experimentally infected

with infectious salmon anaemia virus (ISAV) exhibited an impaired response to PHA and LPS (Dannevig *et al.*, 1993). Studies on immunocompetence and disease susceptibility of juvenile damselfish to neurofibromatosis related tumor formation have revealed that though juvenile fish were more susceptible to disease than adult fish, there was no significant difference in mitogen induced proliferation of lymphocytes between healthy adult and juvenile fish (McKinney and Schmale, 1993).

One of the earliest studies on lymphocyte heterogeneity of fish lymphoid organs was by Etlinger *et al.* (1976) who found that thymocytes responded to Con A, but not to LPS. In contrast lymphocytes from anterior kidney were stimulated by LPS but not by Con A and both mitogens stimulated cells from spleen and peripheral blood. However, Cuchens and Clem (1977) were unable to find a similar distributional dichotomy of lymphocytes in bluegill. Anterior kidney lymphocytes were sorted into separate populations by using rabbit antiserum to bluegill brain tissue. Some heterogeneity was observed in anterior kidney lymphocytes with one population responding to Con A and another population responding to LPS. Warr and Simon (1983) in their study on the heterogeneity of rainbow trout lymphocytes found that lymphocytes from thymus, spleen, head kidney and peripheral blood were capable of some degree of blastogenic response to both Con A and LPS. Even a clear and significant response to Con A of anterior kidney lymphocytes was observed. Caspi *et al.* (1984) observed that in common carp, both peripheral blood and kidney lymphocytes responded to PHA and LPS. Similar results were observed in rainbow trout by Thuvander *et al.* (1990). Faulmann *et al.* (1983) used an improved lymphocyte culture system with a combination of human and channel catfish serum as a culture media supplement and observed that channel

catfish peripheral blood and spleen lymphocytes responded equally well to both LPS and Con A but even their improved culture system failed to stimulate lymphocytes from anterior kidney with either mitogen. According to Tillitt *et al.* (1988), rainbow trout peripheral blood lymphocytes responded better to Con A stimulation than LPS stimulation.

Reitan and Thuvander (1991) reported that in both Atlantic salmon and rainbow trout, lymphocytes from blood, spleen and anterior kidney responded to both LPS and PHA, but peripheral blood and spleen lymphocytes tended to respond better to LPS than PHA. Peripheral blood lymphocytes from spotted gar also responded to both Con A and LPS stimulation (Luft *et al.*, 1994). Daly *et al.* (1995) found that brook trout peripheral blood lymphocytes responded more vigorously to LPS than Con A. European seabass peripheral blood lymphocytes responded well to both Con A and LPS, but spleen lymphocytes did not respond to either Con A or LPS stimulation while head kidney lymphocytes responded better to LPS than Con A stimulation and LPS stimulation of peripheral blood and head kidney lymphocytes were not significantly different from each other (Galeotti *et al.*, 1999). Mitogens Con A and LPS have both been found to be capable of stimulation of lymphocytes isolated from peripheral blood, spleen and head kidney of the experimental groups of fish used in this study. The results of the present study confirms that while lymphocyte heterogeneity exists in Nile tilapia, we cannot be certain that classical mammalian B and T type lymphocytes occupy identifiable anatomical locations in the experimental groups of fish. Warr and Simon (1983) reported similar observations in rainbow trout.

In the present study the stimulation indices are on the whole similar to those observed by Etlinger *et al.*, 1976; Tillitt *et al.*, 1988; Reitan and Thuvander, 1991 and Galeotti *et al.*, 1999. FBS was used as the serum supplement for the assay system. Mitogenic components present in FBS often induce non-specific stimulation of control cultures in the absence of any mitogen which can, in part, obscure the magnitude of the mitogen-induced stimulation (DeKoning and Kaattari, 1992). It has been reported that in some peripheral blood lymphocyte control cultures non-specific ^{3}H-thymidine uptake was as high as 10,000 disintegration per minute (DPM) for $5x10^{5}$ cells (Tillitt *et al.*, 1988) and 45,000 counts per minute CPM for $1x10^{6}$ cells (Etlinger *et al.*, 1976). It has also been reported that blood lymphocytes often showed higher incorporation of ^{3}H-thymidine in control cultures than in experimental cultures leading to relatively low S.I. (Tillitt *et al.*, 1988). According to them, inhibitory factors, such as hormones present in FBS might be responsible for the low S.I. obtained in their study on rainbow trout. In the present study, significantly higher counts were observed in control cultures of blood, spleen and kidney of OBC (AXB) (mean CPM 19945.7, 17782.7 and 15725.1) and URC fish (mean CPM 18393.4, 18726.6 and 19296.5) than the counts of clonal line A (mean CPM 4655.2, 6100.3 and 5582.2) and clonal line B (mean CPM 7614.3, 7904.0 and 9557.5) fish due to non-specific polyclonal lymphocyte proliferation induced by FBS. This might be responsible for low S.I. observed in the OBC (AXB) and URC fish (Appendix 5).

In the present study, lymphocytes were maintained in a normal incubator for four days before they were harvested. Lymphocyte viability was greatly reduced if cultures were maintained for a longer period of time. In salmonids, lymphocyte proliferation

reached a peak response at or before day four in culture when FBS was used in the mitogen stimulation assay but with homologous plasma, proliferation reached a peak response later, at approximately day six (DeKoning and Kaattari, 1992). Therefore, FBS was chosen as the serum supplement over homologous serum or plasma.

The results of this experiment did not reveal any significant difference in lymphocyte proliferation among different groups of experimental fish. There might be differences in polyclonal activation of lymphocytes by mitogens among the different experimental groups of fish when they were infected with pathogen but healthy fish showed no difference in polyclonal activation of lymphocytes. Similar observations were reported in damselfish (McKinney and Schmale, 1993).

CHAPTER 6

SUMMARY AND CONCLUSION

6. Summary and conclusion

The Nile tilapia is one of the important aquaculture species as well as very popular as a laboratory animal. The results of a study on comparison of immune response and disease resistance in clonal lines of Nile tilapia are presented in this thesis. Lymphocyte proliferation using mitogens was also carried out to compare general immunocompetence of these clonal lines.

Clones are completely homozygous for every gene locus therefore, they have potential for fixing superior genes (e.g. disease resistance). Although inbred lines have led to rapid progress and became indispensable in virtually every branch of biomedical science, relatively little research has been done on isogenic and inbred clonal lines of fish in immunology.

In the present study three second generation clonal lines (clonal lines A, B and C) were produced from females of the respective existing clonal lines by meiotic gynogenesis followed by sib mating between females and sex reversed neomales from the same clonal line. Clonal lines D and E were first generation clones produced by meiotic gynogenesis from XX mitotic gynogenetic daughter of XY neofemales. Survival rate at both pigmentation and yolk sac absorption stage was poor in the meiotic gynogenetics as well as in the respective control groups. This might be due to poor egg quality of the females used.

The genetic sex of the clonal individuals from all these clonal lines was also tested by progeny testing using suitable test individuals. No males were observed in any of the clonal lines.

Attempts were made to use microsatellite loci in multiplex reactions to verify the success of the chromosome set manipulations involved in the production of these clonal lines. Seven microsatellite loci were screened for heterozygosity in founder females and donor males used to produce the clonal lines. Based on the evidence of allelic variation data between the founder females and donor males five microsatellite loci (UNH 189, UNH 203, UNH 208, UNH 211 and UNH 228) were selected for verification of clonal lines A, B and E and three loci (UNH 189, UNH 208 and UNH 228) for clonal lines C and D. Pentaplex PCR for clonal lines A, B and E and triplex PCR for clonal lines C and D were attempted. However only triplex and duplex PCR reactions were successful in parentage analysis. Analysis of data from fragment analysis of PCR products revealed that all clonal individuals in all clonal lines inherited maternal alleles. In the experiment, pre-designed primers that were not designed specifically to work in multiplex were used in multiplex PCR. Therefore in some occasions multiplex PCR was not successful. However, the application of polymorphic microsatellite loci for verification of clonal lines was successful.

Though five clonal lines were successfully propagated only clonal lines A and B were used in further studies in immune testing because of insufficient number of fish produced from the other clonal lines. It would have been interesting to see the range of immune response in these clonal lines. An outbred clonal line of fish, OBC (AXB), was produced by crossing female and male from clonal line A and B. An unrelated control (URC) group of Nile tilapia was also used in immune testing.

The specific immune response of the experimental fish was measured following immunisation with a number of different antigens i.e. SRBC, DNP-KLH, TNP-LPS and

vaccination by pathogen *A. hydrophila* strain T4. The kinetics of development of antibody producing cells in peripheral blood, spleen and head kidney were assessed by a PFC or an ELISPOT assay. The serum antibody titre was measured by ELISA assay.

All experimental fish had a similar pattern in the kinetics of the development of PFC or ASC response and serum antibody titres, but the magnitude of these responses differed between experimental groups after immunisation with SRBC, DNP-KLH and TNP-LPS. In general, the specific immune response of clonal line A fish was always significantly higher than that of clonal line B fish. The OBC (AXB) line fish had an intermediate response between clonal lines A and B fish, which was generally significantly lower than clonal line A fish but higher than that of clonal line B fish in most cases. The response of URC fish was lower than that of clonal line A, but this difference was generally not significantly different to clonal line A with a few exceptions. The magnitude of responses seen in OBC (AXB) line and URC fish were very similar.

The experimental groups of fish were vaccinated with heat- killed *A. hydrophila* T4. Clonal line A fish showed a significantly higher serum antibody titre than all other three groups of fish and clonal line B fish showed a significantly lower titre than the other three groups of fish after vaccination against *A. hydrophila* T4. Antibody titres of OBC (AXB) and URC fish were in between those of clonal line A and clonal line B and not significantly different from each other.

The vaccinated and non-vaccinated control fish of the experimental groups were challenged with *A. hydrophila* T4 63 days post-vaccination. The experimental challenge in the vaccinated groups, resulted in clonal line A having a significantly lower level of

mortality than clonal line B and OBC (AXB) fish. Clonal line B fish had significantly higher mortality than clonal line A, OBC (AXB) and URC fish. OBC (AXB) fish mortality was intermediate, significantly higher than clonal line A but significantly lower than clonal line B. Mortalities in clonal line A and URC were not significantly different. In the non-vaccinated group, clonal line B had significantly higher mortality than clonal line A. OBC (AXB) and URC fish had mortalities in between clonal line A and B but not significantly different from either group. These results showed the inverse relationship between antibody production and susceptibility to disease. Similar results were found in mouse susceptibility to *Trypanosoma cruzi* by Kierszenbaum and Howard (1976) and in carp susceptibility to *Trypanoplasma borreli* (Wiegertjes *et al.*, 1995a,b). The response of OBC (AXB) indicate that in an outbred line, disease resistance can be increased to an intermediate level, between the resistance of the two parents, by crossing between a high and a low disease resistant line of fish. If more clonal lines are available, immune testing may identify even higher responder clonal lines. An outbred line of fish, produced from crossing between two high responders may have significantly increased disease resistance in that line.

Lymphocytes isolated from peripheral blood, spleen and head kidney of the experimental groups of fish were stimulated with classical T-cell mitogen Con A and B cell mitogen LPS. Con A and LPS both stimulated lymphocytes from all three lymphoid tissues tested in all the experimental groups of fish. The results of the present study confirms that while lymphocyte heterogeneity exists in Nile tilapia, we cannot be certain that classical mammalian B and T type lymphocytes occupy identifiable anatomical locations in the experimental groups of fish. Warr and Simon (1983) reported similar

observations in rainbow trout. Though S. I. of lymphocytes from different tissues of the experimental groups of fish to Con A or LPS were different from each other but differences were not always significant. This study could not correlate the polyclonal activation of lymphocytes of healthy fish to disease resistance in the same clonal line. McKinney and Schmale (1993) reported similar results in juvenile damselfish. The specific immune responses to antigens of these experimental groups of fish discussed in Chapter 3 and 4 was different from the response of lymphocytes from healthy fish to polyclonal activation to mitogens, discussed in Chapter 5.

The importance of specific antibodies for protection against pathogens does not negate the role of cell mediated immune responses following immunisation. Indeed, the production of antibody, resulting from proliferation of primary effector cells and their differentiation into both specific antibody secreting cells and specific memory cells, is often a complex process requiring cell co-operation and the production of many cytokines able to promote these events. Smith *et al.* (1980) correlated the production of macrophage inhibition factor (MIF) in response to specific antigen with resistance in vaccinated brown trout. The production of a cytokine macrophage activating factor (MAF) able to up regulate non-specific killing mechanisms following antigen stimulation of primed leucocytes was reported by Marsden *et al.*, 1994 and has important implications for the killing of bacterial pathogens. A similar study involving Nile tilapia clonal lines A and B and OBC (AXB) may be useful in understanding the underlying mechanism of differences in their specific immune responses.

Available information on the specific response of fish to pathogen is mainly based on the response to experimental administration of antigen of typical strains grown *in vitro*.

This information may not be directly applicable to the response of fish to a natural infection by atypical strains for two reasons. Firstly, there may be a difference in the antigen expression of pathogens grown *in vivo* and *in vitro* (Garduno *et al.*, 1993). Infection due to atypical *A. salmonicida* is known to produce different pathological features than typical strains (Laxdal, 1989; Austin and Austin, 1993). Secondly, different factors related to stress such high stocking density, pollutants in water, diet, seasonal fluctuation in water temperature etc. are encountered under farming conditions rather than under controlled experimental conditions. Stressful circumstances are known to depress certain aspects of immune responsiveness. The stress response involves physiological and behavioural reactions, which may help the fish to adapt to a new situation but if the stress is severe or prolonged, it may exceed the capacity of the fish to adjust resulting in a general breakdown of immune system (Ellis, 1988). Experimental challenge with atypical *A. salmonicida* demonstrated genetic variation in resistance between carp strains of different geographical origins (Van Muiswinkel *et al.*, 1990). Differences in the survival of these fish under farming conditions (Wiegertjes *et al.*, 1995d) was correlated to the results of the experimental challenge (Wiegertjes *et al.*, 1993a). On the other hand Ehlinger (1977) found that a strain of brook trout selected against furunculosis susceptibility was in fact very susceptible to gill disease. Therefore, performances i.e. survival and growth of the two clonal lines under typical farming conditions or by exposing them to different pathogens as well as to different strains of the same pathogen would reveal the true nature of disease resistance. *Streptococcus iniae* can be chosen as pathogen for future studies on immunological responses in these clonal lines. The problems of streptococcal disease is worldwide causing huge

economic losses in aquaculture sector. This pathogen has emerged as a serious threat to tilapia culture. This is also pathogenic to human and transmitted vertically (Muzquiz *et al.*, 1999; Shoemaker *et al.*, 2001).

From the present study the possible effects of sex on immune responses can not be readily explained, because all fish in the two clonal lines and OBC (AXB) were females while only the URC group had both males and females. However, with a study on chicken Scott *et al.* (1994) found better antibody response in male chicks, and suggested a possible sex effect on immune response. Similar studies on all female and all male sib clonal lines in this species (produced from XY neofemale by mitotic gynogenesis in the first generation and subsequent meiotic gynogenesis or androgenesis or from XY male by androgenesis to produce the first generation and subsequent meiotic gynogenesis or androgenesis in second generation) may reveal any possible effect of sex on immune response.

The specific immune responses of the clonal lines were less variable between individuals compared to the URC fish, which had higher inter individual variation. This suggests that clonal lines are genetically highly uniform.

The most important aim of selective breeding is the overall resistance to pathogens. Selective breeding has increased resistance to furunculosis in brook trout and brown trout (Ehlinger *et al.*, 1977), to dropsy in common carp (Kirpichnikov *et al.*, 1979) and to *A. salmonicida* in rainbow trout (Fevolden *et al.*, 1992) and in inbred common carp (Wiegertjes *et al.*, 1995d). Hybrids of striped bass *Morone saxatilis* and white bass *M.chrysops* are more resistant to disease than the parental species (Anderson *et al.*, 2001). Selective breeding has also increased resistance against furunculosis, infectious

salmon anaemia and infectious pancreatic necrosis in Atlantic salmon and marker assisted selection for resistance against viral diseases in rainbow trout has been reported (reviewed by Midtlyng *et al.*, 2002).

Clonal lines A and B can used in future as suitable experimental fish to study the immune response to intracellular and extracellular pathogens. as well as to investigate segregation and linkage analysis of class I and class II major histocompatibility genes. Genetic variation is present and fixed in these clonal lines. The segregation pattern of these genes can be followed in F1 and F2 generations produced by crossing these two lines.

Clonal lines have been a valuable tool in demonstrating genetic variation in immune response parameters in the experimental groups of fish because of having genetically uniform animals in the clonal lines. Further studies would be necessary to make progress in achieving genetic improvement in disease resistance in this population. Such studies may involve testing more clonal lines by using some of the parameters tested and developed in this study for indirect selection for immune response and selecting the best clonal lines for production of F1 and F2 generations. More clonal lines might suggest limited numbers of genotypes or markers and simple inheritance of disease resistant genotypes. The segregation pattern of these genotypes or loci (e.g. microsatellite loci) can be followed in the subsequent generations produced by crossing these clonal lines. If full resistant and susceptible offspring can be detected, search for quantitative trait loci (QTL) can be initiated. Once identified, these QTL can be used to screen individuals to be selected and possibly used as brood stock to improve disease resistance in Nile tilapia.

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APPENDICES

Appendix 1. Serum anti-SRBC titres of the experimental groups of fish.

Days post-Immunisation	Clonal line A		Clonal line B		OBC (AXB)		URC	
	Immunised	Non-immunised	Immunised	Non-immunised	Immunised	Non-immunised	Immunised	Non-immunised
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
7	7.5 ± 0.8	2.7 ± 0.2	6.2 ± 0.6	2.3 ± 0.2	7.7 ± 1.6	2.3 ± 0.1	8.3 ± 1.8	2.2 ± 0.1
14	12.0 ± 0.7	2.7 ± 0.2	8.8 ± 1.0	2.2 ± 0.1	10.5 ± 1.6	2.3 ± 0.1	10.8 ± 1.8	2.4 ± 0.2
21	9.1 ± 1.0	2.2 ± 0.1	5.3 ± 0.7	2.2 ± 0.1	8.1 ± 1.6	2.0 ± 0.0	9.1 ± 1.0	2.2 ± 0.1
28	8.1 ± 1.0	1.7 ± 0.2	3.8 ± 0.6	1.7 ± 0.2	6.8 ± 1.9	1.7 ± 0.2	7.8 ± 1.4	1.7 ± 0.2

Appendix 2. Serum anti-DNP titres of the experimental groups of fish.

Days post-Immunisation	Clonal line A		Clonal line B		OBC (AXB)		URC	
	Immunised	Non-immunised	Immunised	Non-immunised	Immunised	Non-immunised	Immunised	Non-immunised
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
7	11.2 ± 0.2	2.3 ± 0.1	8.3 ± 0.4	2.1 ± 0.1	10.1 ± 0.9	2.3 ± 0.1	10.1 ± 0.3	2.3 ± 0.1
14	16.0 ± 0.4	2.3 ± 0.2	12.4 ± 0.3	2.3 ± 0.1	13.1 ± 0.8	2.2 ± 0.1	16.6 ± 0.7	2.3 ± 0.2
21	18.0 ± 0.5	2.1 ± 0.1	12.7 ± 0.5	2.0 ± 0.1	16.3 ± 0.4	2.2 ± 0.1	17.4 ± 0.8	2.1 ± 0.1
28	21.0 ± 0.5	2.1 ± 0.1	13.5 ± 0.4	2.2 ± 0.1	17.0 ± 0.4	2.3 ± 0.2	18.8 ± 0.7	2.2 ± 0.1
35	17.8 ± 0.5	2.3 ± 0.1	11.1 ± 0.2	2.2 ± 0.1	13.6 ± 0.8	2.1 ± 0.1	16.0 ± 0.7	2.1 ± 0.1
42	12.9 ± 0.3	2.0 ± 0.0	8.3 ± 0.3	2.2 ± 0.1	11.2 ± 0.4	2.0 ± 0.0	11.7 ± 0.4	2.0 ± 0.0
49	11.3 ± 0.4	1.8 ± 0.3	5.6 ± 0.3	1.6 ± 0.3	8.8 ± 0.6	2.0 ± 0.2	9.7 ± 0.8	1.6 ± 0.3
56	9.7 ± 0.3	1.8 ± 0.3	3.1 ± 0.2	1.2 ± 0.3	6.5 ± 0.6	1.7 ± 0.3	7.8 ± 0.6	1.2 ± 0.3

Appendix 3. Serum anti-TNP titres of the experimental groups of fish.

Days post-Immunisation	Clonal line A		Clonal line B		OBC (AXB)		URC	
	Immunised	Non-immunised	Immunised	Non-immunised	Immunised	Non-immunised	Immunised	Non-immunised
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
7	12.7 ± 0.3	2.3 ± 0.1	8.8 ± 0.4	2.1 ± 0.1	10.7 ± 0.4	2.3 ± 0.1	10.8 ± 0.6	2.3 ± 0.1
14	16.6 ± 0.2	2.2 ± 0.1	11.7 ± 0.2	2.1 ± 0.1	12.3 ± 0.4	2.3 ± 0.1	13.4 ± 0.7	2.2 ± 0.1
21	12.9 ± 0.1	2.2 ± 0.1	10.2 ± 0.1	2.2 ± 0.1	12.1 ± 0.4	2.3 ± 0.1	12.2 ± 0.7	2.3 ± 0.2
28	11.8 ± 0.3	2.4 ± 0.2	9.2 ± 0.3	2.1 ± 0.1	10.8 ± 0.6	2.2 ± 0.1	11.5 ± 0.6	2.1 ± .01
35	11.3 ± 0.3	2.3 ± 0.1	7.4 ± 0.3	2.2 ± 0.1	10.5 ± 0.6	2.2 ± 0.1	10.3 ± 0.6	2.7 ± 0.1
42	10.4 ± 0.3	2.1 ± 0.1	5.3 ± 0.2	2.2 ± 0.1	9.7 ± 0.4	2.1 ± 0.1	9.3 ± 0.6	2.3 ± 0.1
49	10.0 ± 0.3	1.5 ± 0.3	3.5 ± 0.2	1.5 ± 0.3	8.3 ± 0.4	1.9 ± 0.2	8.5 ± 0.6	1.8 ± 0.3
56	9.1 ± 0.3	1.5 ± 0.3	3.0 ± 0.2	1.5 ± 0.3	6.7 ± 0.4	1.7 ± 0.2	7.2 ± 0.7	1.7 ± 0.2

Appendix 4. Serum anti-*A. hydrophila* titres of the experimental groups of fish.

Days post-vaccination	Clonal line A		Clonal line B		OBC (AXB)		URC	
	Vaccinated	Non-vaccinated	Vaccinated	Non-vaccinated	Vaccinated	Non-vaccinated	Vaccinated	Non-vaccinated
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
14	12.8 ± 0.5	2.2 ± 0.1	8.3 ± 0.4	2.0 ± 0.0	12.0 ± 1.5	2.3 ± 0.1	12.7 ± 1.0	2.1 ± 0.1
28	16.8 ± 0.7	2.3 ± 0.1	11.6 ± 0.7	2.2 ± 0.1	14.3 ± 1.5	2.3 ± 0.2	15.1 ± 1.0	2.2 ± 0.1
42	16.3 ± 0.7	2.3 ± 0.1	7.1 ± 0.4	2.2 ± 0.1	11.4 ± 1.2	2.2 ± 0.1	11.3 ± 0.9	2.1 ± 0.1
56	14.1 ± 0.7	1.8 ± 0.3	4.1 ± 0.4	1.8 ± 0.3	9.8 ± 1.2	1.7 ± 0.3	10.3 ± 0.9	1.8 ± 0.2

Appendix 5A. Counts per minute of stimulated and non-stimulated lymphocytes in clonal line A.

Fish	Replicate	Stimulated (CPM)						Non-stimulated (CPM)		
		Con A			LPS					
		Blood	Spleen	Head kidney	Blood	Spleen	Head kidney	Blood	Spleen	Head kidney
1	1	43327.20	42940.20	39717.20	26861.30	43334.50	26514.10	9327.68	4244.60	4489.93
	2	47053.30	43084.70	27248.10	37981.10	52405.60	19361.70	3981.04	4261.76	3971.75
2	1	46541.30	29910.90	22250.70	24340.80	40432.30	13825.70	3399.92	4652.12	3032.06
	2	36739.00	44197.40	21685.00	26795.20	32653.90	17611.70	5599.22	3677.63	6478.45
3	1	63060.70	60355.30	24942.70	26512.60	34934.50	17561.60	2644.00	2221.18	2498.34
	2	43099.70	65390.80	28946.00	32229.10	43201.50	22928.50	3702.64	2692.40	2498.34
4	1	44032.00	54168.70	25464.20	10697.50	26602.50	1311.77	3266.32	1255.46	1188.79
	2	51151.20	68321.20	22432.60	10798.36	26520.66	1673.24	4428.09	4119.63	1311.73
5	1	22632.30	10401.70	946.63	33108.50	17501.60	46870.30	822.16	2970.85	587.07
	2	20807.50	6618.93	1035.75	10269.70	14601.60	28549.40	785.78	1477.97	10152.40
6	1	33362.80	105827.00	117543.00	5153.79	7160.77	8476.19	728.95	645.10	10152.40
	2	10990.60	52439.00	20553.20	4748.48	7520.67	5195.00	21584.70	722.90	56558.40
7	1	6991.64	10941.70	4049.94	26066.80	34565.80	18257.90	10043.90	13281.80	30221.00
	2	6602.22	9992.00	3975.65	9827.90	12464.30	12631.10	12913.30	55825.70	12837.90
8	1	91515.70	6312.86	2217.81	3479.00	7711.87	7024.38	14570.30	61052.40	10152.40
	2	38638.10	2139.29	1365.23	3338.90	8160.73	66115.00	14570.30	18121.70	15860.20
9	1	1477.92	925.98	1418.04	3086.30	6617.21	2841.74	1450.77	1591.88	336.92
	2	1413.26	745.92	641.85	2830.97	4270.19	2377.16	1001.39	2884.44	443.04
10	1	1027.20	224.46	160.40	3044.41	7806.45	1091.13	1098.16	1414.82	2567.33
	2	2559.68	320.27	145.14	2875.60	4623.10	1620.28	1058.40	658.88	2567.33
11	1	2404.72	2618.43	1175.23	4503.59	6602.99	1555.99	243.96	5632.78	1530.32
	2	1690.00	1255.08	685.41	5058.07	5140.94	1806.51	2623.12	5720.04	1342.96
12	1	1883.72	1205.61	312.93	7168.70	34607.80	1022.71	3732.80	5182.99	3840.43
	2	5586.68	2419.80	650.84	6986.83	19694.00	4282.91	5519.67	1116.98	8132.24
13	1	56077.60	34227.50	7421.63	27843.70	60083.90	72888.10	2017.95	1418.21	2025.59
	2	48048.70	23796.80	3620.93	13446.70	41714.60	46553.10	1295.64	1086.94	1815.10
14	1	35084.80	16237.30	2461.23	10153.20	31992.20	19706.00	1724.06	4213.58	1792.52
	2	32163.70	16100.20	2790.35	8952.30	35825.40	20242.80	1209.83	2602.27	1947.50
15	1	77198.90	36408.90	10885.10	5111.12	39277.20	103417.00	198.84	583.00	690.51
	2	61995.20	17675.90	8640.25	3881.64	20870.30	77165.00	267.90	611.86	753.60
16	1	37023.80	31645.30	1638.55	20733.90	18285.70	39596.30	305.05	444.03	907.13
	2	35354.00	21195.70	5322.40	2252.79	16022.10	37554.20	366.53	662.15	1441.28
17	1	4534.63	1058.14	1675.51	603.45	940.65	2863.76	4133.50	4416.46	4718.66
	2	4026.22	1163.42	1001.70	996.45	849.62	3103.14	4815.61	4622.50	1107.01
18	1	16438.10	3161.73	4796.69	2289.40	726.87	3103.98	14550.80	4872.35	939.19
	2	8113.95	2067.49	1816.18	1393.41	804.67	6737.55	15863.30	5467.27	978.20
19	1	7566.25	1557.06	1408.60	210.75	567.38	4147.57	2443.60	2987.17	1490.07
	2	6953.02	1940.66	1913.39	2226.52	554.48	6303.59	2179.21	1834.06	2978.03
20	1	2266.74	4715.99	4778.33	1350.24	4752.57	8629.77	2593.30	1161.23	4532.59
	2	1219.65	1525.27	4792.19	1108.07	4761.50	8071.53	3147.93	1604.68	2418.41

Appendix 5B. Counts per minute of stimulated and non-stimulated lymphocytes in clonal line B.

Fish	Replicate	Stimulated						Non-stimulated		
		Con A			LPS					
		Blood	Spleen	Head kidney	Blood	Spleen	Head kidney	Blood	Spleen	Head kidney
1	1	28940.90	43301.80	56714.50	32689.00	61544.20	45751.40	6569.92	1979.38	1828.46
	2	51846.30	54867.70	60538.30	25511.80	85819.80	42835.90	2234.34	2595.85	2068.38
2	1	48457.80	63009.60	61500.80	21088.20	62083.30	23474.80	2564.44	4366.16	2103.63
	2	49626.30	56269.30	58470.40	22086.60	64565.70	27394.00	2174.45	2130.41	3496.84
3	1	67867.40	71889.70	59821.30	26802.90	63324.50	24728.50	2146.91	1434.29	810.94
	2	52975.30	68326.50	57290.90	28420.30	106651.00	36041.30	1264.66	811.59	438.64
4	1	49656.60	58040.90	55275.10	18360.40	31167.10	17612.10	5466.02	4545.05	5105.60
	2	52528.50	54707.10	56984.70	17779.30	30990.25	18629.30	5469.00	4434.04	5098.10
5	1	28944.10	9715.91	1835.38	11790.20	5939.89	12365.80	910.25	1092.29	1301.50
	2	38620.70	10092.00	2272.90	11980.30	3538.23	9537.61	2145.36	886.82	1720.04
6	1	53565.30	11763.30	2608.88	18360.80	42668.80	16015.90	1489.32	1103.32	1252.87
	2	36100.00	6205.27	5796.22	38323.90	24832.30	14192.80	1292.31	1305.32	1111.83
7	1	65702.90	13329.80	4828.53	14956.40	24031.70	18851.60	50543.20	14011.20	1111.83
	2	109915.00	14736.50	5927.75	5913.56	18983.10	26296.60	13116.10	16595.20	17634.30
8	1	170866.00	23310.70	4382.20	4795.71	16653.20	75009.80	13116.10	21554.80	1252.87
	2	274978.00	25007.40	6906.34	4354.69	17416.30	37341.70	13116.10	1305.32	18950.80
9	1	4103.80	1318.05	2685.73	14521.60	10077.70	15593.20	21551.40	23892.50	34013.20
	2	2330.12	1355.21	2719.36	6995.16	3811.18	13008.50	18393.90	33155.50	31316.80
10	1	1293.64	4069.09	2871.57	8324.06	3763.83	11152.80	19070.00	39623.10	38280.20
	2	1645.51	1788.95	2806.16	7636.58	3723.04	13214.70	18920.10	30570.60	38931.30
11	1	3403.08	5278.18	1406.18	14696.60	5808.39	8060.67	10686.40	4984.76	7042.10
	2	2575.06	4241.99	1451.55	9251.55	4035.22	6192.38	4782.28	5212.89	7735.69
12	1	2577.73	2297.21	505.18	12554.10	4807.71	4817.27	4897.18	13736.90	8247.39
	2	3553.29	1806.92	1100.86	14457.90	3694.16	4595.88	5219.88	8642.56	10735.10
13	1	2725.75	3024.06	1768.84	5958.97	38763.40	3733.82	776.39	1134.21	9431.01
	2	2104.46	3075.45	1580.96	7659.44	36923.60	4431.77	717.78	764.13	28605.50
14	1	3979.91	4603.17	2347.35	8944.77	43951.40	5953.61	887.60	558.56	5502.08
	2	4723.93	3322.98	3710.36	6732.95	37036.80	8784.00	1296.27	1007.16	4495.73
15	1	3554.70	1605.92	2726.89	6840.86	42268.20	10485.50	5476.93	2580.55	2527.80
	2	3718.20	1711.88	2384.17	4246.94	34493.00	9315.42	2395.63	2329.58	2041.93
16	1	7825.33	16580.90	18046.90	11072.30	26954.90	11001.60	2230.17	2416.52	2695.77
	2	3015.11	1956.62	1946.69	11052.60	34195.00	3440.58	2739.04	2170.31	8952.23

Appendix 5C. Counts per minute of stimulated and non-stimulated lymphocytes in OBC (AXB).

Fish	Replicate	Stimulated						Non-stimulated		
		Con A			LPS					
		Blood	Spleen	Head kidney	Blood	Spleen	Head kidney	Blood	Spleen	Head kidney
1	1	58153.70	39625.40	24715.50	34116.60	20223.80	2445.24	3784.74	3338.27	2884.35
	2	38250.80	48411.90	23975.40	40128.00	44355.70	2365.99	7042.67	2618.05	1965.67
2	1	40110.50	38642.60	31452.60	22197.00	24530.10	2688.52	6792.53	1965.44	1346.74
	2	44748.20	47225.30	30891.00	17804.20	26371.80	1886.19	5352.14	5135.19	1261.94
3	1	58484.00	48659.70	20343.70	19980.00	28195.00	2191.35	3591.73	674.83	433.88
	2	39332.80	37186.60	15110.60	19413.60	32611.50	1622.46	3220.73	593.41	578.24
4	1	56954.70	66221.40	37679.70	23177.9	25307.80	43619.90	11439.20	9690.68	7955.10
	2	46856.40	38807.70	31859.90	26169.8	25540.90	33272.80	11640.05	9257.64	8077.90
5	1	24911.60	19863.70	23024.80	25337.30	20567.50	54754.10	29823.50	39845.80	30402.60
	2	18447.20	25059.10	39352.20	27338.40	26283.20	60344.00	69988.90	32974.80	27799.50
6	1	11431.30	16635.10	20380.20	18792.90	22779.80	49774.10	44375.70	20462.90	25288.10
	2	12779.20	17511.30	18086.70	20122.30	34170.80	22892.10	40455.70	57940.40	24697.60
7	1	21211.50	40105.40	21737.70	20386.40	37639.70	64121.70	87004.90	25735.80	32915.00
	2	14270.80	17979.50	25969.80	23119.70	40162.60	72653.20	69277.50	42162.20	39293.00
8	1	9485.11	13722.20	14563.00	20296.10	34646.40	75200.80	114182.00	38182.50	31798.50
	2	13019.70	19430.00	18892.30	16179.60	42319.00	59117.80	32197.90	68845.10	38193.00
9	1	28702.90	34186.80	11543.00	10467.50	4105.76	13515.90	10122.10	3021.16	6907.61
	2	15439.90	12322.00	16439.60	6113.91	1763.13	16301.20	7978.29	3537.68	6635.20
10	1	6164.36	6550.96	4586.94	5936.01	874.19	8219.79	4269.14	10171.60	7065.53
	2	6626.27	8877.22	3824.49	7813.45	615.16	8643.41	3516.05	10162.70	59921.60
11	1	19404.60	28719.50	33232.16	13098.90	2314.80	13515.90	28772.40	6598.22	4647.22
	2	10234.50	9220.54	14684.80	4832.76	2012.83	29675.50	13883.80	6464.94	4799.77
12	1	6144.40	4663.17	6320.21	3605.33	2628.35	16822.80	6614.42	16782.70	4079.58
	2	8216.25	4955.87	6383.87	3946.78	1605.91	19311.40	7125.00	8839.99	4136.60
13	1	21429.90	24115.50	18474.80	25934.80	24228.80	17484.00	9842.91	57909.40	36782.90
	2	39639.90	51793.20	56948.70	44156.90	57708.20	22618.10	37996.20	62851.50	22963.90
14	1	31363.50	36941.20	31594.60	25211.80	48936.70	27513.60	21032.10	13044.60	48870.80
	2	35733.10	41693.50	45530.10	40586.10	69800.70	37917.20	18222.30	15436.70	27085.00
15	1	21875.30	23826.00	14502.90	38498.80	25308.80	16194.40	15532.90	32375.90	20721.00
	2	24059.00	28977.50	17048.20	52615.20	26302.90	15081.10	19382.60	62855.20	14585.70
16	1	31574.40	37348.70	40339.50	44484.60	51188.40	28154.20	18038.70	14379.60	25347.60
	2	27523.40	38227.60	37505.40	99309.70	77739.10	43619.90	21162.50	13261.50	44250.30
17	1	7935.00	5665.29	1494.61	19009.30	43171.40	33272.80	1889.78	2555.72	1483.16
	2	8750.30	4662.83	1512.81	12685.00	8177.05	7352.38	1741.26	803.97	1186.74
18	1	1635.67	4588.70	1461.89	16768.40	14308.40	12216.40	1086.22	747.53	2007.18
	2	2988.54	5444.42	1386.80	17834.60	12190.90	10153.30	1510.46	1245.64	2007.18
19	1	967.41	4267.73	716.79	46332.90	24345.60	15859.10	2337.03	1993.52	1626.04
	2	1548.30	3748.76	984.42	21195.40	11340.20	14598.70	2553.71	3680.75	2286.17
20	1	1779.15	8811.94	1201.09	20298.40	9635.97	13698.90	1827.36	1056.81	2127.03
	2	2897.48	9763.79	3599.71	31659.90	10961.20	16090.20	1220.70	2108.14	2590.11

Appendix 5D. Counts per minute of stimulated and non-stimulated lymphocytes in URC.

Fish	Replicate	Stimulated						Non-stimulated		
		Con A			LPS					
		Blood	Spleen	Head kidney	Blood	Spleen	Head kidney	Blood	Spleen	Head kidney
1	1	43747.20	20391.60	30384.80	29255.30	44981.10	64671.30	7943.31	15019.90	13034.20
	2	33690.00	29325.10	20685.00	32972.40	59621.40	78604.40	8294.17	16154.60	17605.50
2	1	22695.90	25939.10	26641.00	30052.70	72698.40	67075.90	19011.70	8416.04	16130.20
	2	50148.80	34809.00	25949.60	28894.40	106309.00	98096.50	11427.40	12615.80	11810.10
3	1	38931.90	42434.70	37723.30	110617.00	60962.20	70676.30	5111.01	5794.05	2394.91
	2	33051.00	44434.90	26564.30	9707.95	55708.50	89821.70	4466.11	4110.65	3413.79
4	1	30642.90	28445.00	29645.70	11261.00	50059.30	95632.20	5472.84	1570.47	4534.25
	2	29863.70	23248.90	28037.50	24185.50	101670.00	123705.00	7373.13	2271.83	6970.57
5	1	44201.50	18516.10	25714.40	15474.60	123840.00	56861.00	32483.90	31832.10	22959.60
	2	29847.40	20584.20	26485.70	16633.40	27915.90	6173.86	17728.50	20141.50	21498.50
6	1	42724.90	15678.20	23951.00	13484.80	46643.90	5882.47	17728.50	17419.20	22959.60
	2	18315.80	70028.30	68037.00	24106.05	59217.20	10155.60	29654.10	18920.90	21498.50
7	1	13952.20	20507.70	15359.60	23560.10	230405.00	79798.70	55890.70	14738.40	16128.10
	2	28421.00	18784.20	13847.30	13003.80	5115.43	29383.60	14562.40	14738.40	16128.10
8	1	28492.70	14181.50	33553.90	17486.40	4849.80	27507.60	9790.33	40419.20	22879.90
	2	18627.00	40443.50	57372.20	25190.40	5859.89	34615.30	11717.90	18693.40	23188.90
9	1	8648.47	9576.62	1577.75	1214.20	6277.83	9143.61	1836.53	400.15	1140.02
	2	12778.20	5385.76	1335.89	583.00	3289.22	1987.61	1390.89	545.93	4221.75
10	1	3568.11	3995.29	1046.88	286.09	4613.24	1538.50	1026.49	2335.04	8487.48
	2	3460.84	2903.72	778.31	215.54	5414.28	1659.88	551.73	1220.97	4425.09
11	1	16295.40	10532.00	2829.42	742.25	6177.83	3711.16	5813.22	595.34	1029.88
	2	8903.31	3956.52	2255.73	912.79	4100.74	1916.13	1015.33	736.25	867.27
12	1	2027.44	2495.06	3372.19	1410.78	4292.62	1593.32	981.90	1382.67	1045.62
	2	1581.79	4369.39	2801.65	3700.89	4202.93	1472.20	1089.51	1355.81	1045.62
13	1	11979.80	12557.90	71003.00	65576.90	94885.70	29569.30	42066.60	42098.70	51673.00
	2	10813.40	9458.17	30174.60	30644.70	76020.19	25871.10	41786.10	37397.10	68189.90
14	1	15723.50	11203.20	40003.90	29250.50	76530.50	27516.30	40863.40	89564.30	55894.30
	2	14419.70	16221.00	86694.60	24124.10	50744.00	25524.50	38577.40	54660.90	43949.30
15	1	22062.50	8426.24	44396.80	13690.00	29134.40	24968.90	27939.30	32371.40	35396.60
	2	21578.90	8051.55	34376.80	38701.30	29165.60	26275.10	38987.60	39803.90	30491.90
16	1	22001.10	13601.00	51767.00	33251.20	29201.60	32741.20	48224.90	23844.10	36791.30
	2	14086.10	13794.50	26485.70	32483.40	36951.10	25632.00	37782.90	28083.70	29704.30