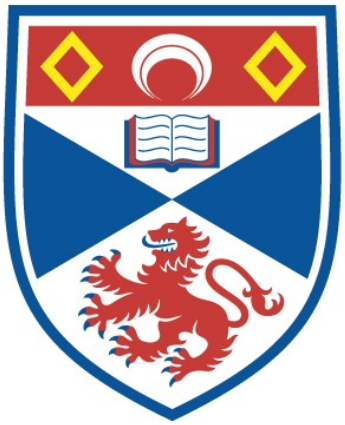
A COMPARISON OF SOME ASPECTS OF THE IMMUNE SYSTEM IN DIPLOID AND TRIPLOID ATLANTIC SALMON

Anne Louise Langston

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1999

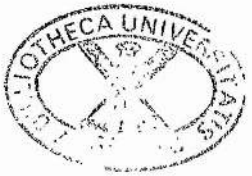
A Comparison of Some Aspects of the Immune System in Diploid and Triploid Atlantic Salmon

Anne Louise Langston

BSc (Hons) Ocean Science, University of Plymouth, 1993.
MSc Applied Fish Biology, University of Plymouth, 1994.

A thesis submitted for the Degree of Doctor of Philosophy, University of St Andrews.

March 1999.



Declarations

I, Anne Langston, hereby certify that this thesis, which is approximately words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted n any previous application for a higher degree.

Date 4th Aug '99 Signature of Candidate

I was admitted as a research student in January, 1995 and as a candidate for the degree of Doctor of Philosophy in January 1995; the higher study for which this is a record was carried out at the FRS Marine Laboratory, Aberdeen between 1995 and 1998.

Date 4th Aug 199 Signature of Candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date 4th Aug '99 Signature of Supervisor

In submitting this thesis to the University of St. Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any bona fide library or research worker.

Date 4th Aug '99 Signature of Candidate

ACKNOWLEDGEMENTS

I would like to express my sincerest thanks to anyone and everyone who has helped with the work in this thesis and my deepest apologies if they are not mentioned by name. There have been so many helpful and supportive people I'm bound to forget some.

Thanks to...

- Tony Ellis and Ray Johnstone for everything (which is more than I can list here)
- Everyone in the immunology lab (including some who've moved on) and other people within the FCB for general help, advice, guidance, motivation, entertainment and friendship. Especially, Ian Bricknell, Julie King, Andy Barnes and Ricky Butler.
- Nadine Veyret for her role in the analysis of the samples within Chapter 9,
- Susan McGruer for help with the mind-melting task of counting and measuring blood cells and accompanying me on a snowy trip to Norway.
- To Rob Fryer for helping me to wade through the statistical mire.
- Philip MacLachlan for production of the triploid fish. To Philip, Sally Clements and other members of staff at the Fish Cultivation Unit in Aultbea for rearing many of the fish used in this thesis, monitoring experiments, aiding with sampling and helping me to improve my sampling skills.
- Tone Knappskog, Frode Oppedal, Tom Hanson and Geir-Lasse Taranger for allowing me to do experiments in Norway, taking samples and looking after my fish. To many others in Matre and Bergen for helping to keep me sane during my visits.

But mostly, a million thanks to Timbo for everything thesis associated or otherwise.

Table of Contents

List of Figures

List of Tables

List of Publications

Chapter 5

Langston, A.L.; Bricknell, I. R. and Ellis, A. E. **1998** *The Iron Binding Capacity of Peripheral Blood Leucocyte Lysates of Atlantic salmon* (Salmo salar) The proceedings of the 1st Symposium of Methodologies in Fish Disease Research

List of Abbreviations

%	percentage
%sat	percentage transferrin saturation
µg	microgram
µm	micrometre
2n	diploid
3n	triploid
A	absorbance
ACH50	alternative pathway complement activity
ACP	alternative complement pathway
ANOVA	analysis of variance
CCP	classical complement pathway
CH50	classical pathway complement activity
D	diploid
DMSO	dimethyl sulphoxide
EA	erythrocyte antibody suspension
EDTA	ethylene diamine tetraacetic acid
FCS	fetal calf serum
FRS	Fisheries Research Services
g	gram
GVB	gelatin veronal buffer
HBSS	hanks buffered salt solution
HEWL	hen egg white lysozyme
hr	hour
kg	kilogram
l	litre
LPS	lipopolysaccharide
m	metre
M	molar
MANOVA	multivariate analysis of variance
mg	milligram
min	minute
ml	millilitre

mOD	milli optical density
MS222	tricane methane sulphonate
NBT	nitro blue tetrazolium
nm	nanometre
No. Obs	number of observations
°C	degree centigrade
OD	optical density
PBL	peripheral blood leucocyte
PBS	phosphate buffered saline
PCV	packed cell volume
Pen strep	penicillin/streptomycin
PMA	phorbol myristate acetate
RaRBC	rabbit red blood cell
rb	reagent blank
s	sample
sb	sample blank
SOD	superoxide dismutase
SRBC	sheep red blood cell
Std Dev	standard deviation
Std Err	standard error
T	triploid
TAT	total antitrypsin activity
TI	total serum iron content
TIBC	total iron binding capacity
UIBC	unsaturated iron binding capacity
v/v	volume/volume
VBS	veronal buffered saline
w/v	weight/volume
w/w	weight/weight
xg	centrifugal force
ΔA	change in absorbance
α2m	alpha-2 macroglobulin

ABSTRACT

This study investigated the differences imposed by the extra set of chromosomes on the immune system of a triploid animal. Various haematological and immunological parameters in diploid and triploid sibling Atlantic salmon (*Salmo salar*) were compared. Haematocrits were usually the same in diploid and triploid siblings. Erythrocytes and leucocytes were larger and fewer in number in triploid siblings. However, they were not larger or fewer in number by a factor of one third. The proportions of the different leucocyte populations did not differ between diploids and triploids. This was the case for 3 families of diploids and triploids. Non-specific, humoral factors were not consistently different between diploids and triploids. From an investigation of 3 families it was found that there may be family influences on how diploids and triploids compare. A study of the kinetics of responsiveness of non-specific, humoral factors showed that triploids did differ from diploids in the speed of the immune reaction or in the rate of recovery. However, the profile of the differences between diploids and triploids varied between parameters. The rate of increasing agglutinating titres in response to treatment with a commercial vaccine did not differ between diploids and triploids. However, triploids did have a higher average titre after 24 weeks in comparison to diploids. Non-specific, cellular parameters did differ between sibling diploids and triploids. Triploid kidney macrophages had an increased phagocytic capacity but the proportion of phagocytic cells did not differ. Intracellular respiratory burst activity was not different between diploid and triploid kidney macrophages. Diploids and triploids did not respond differently to predator stress and there was no difference in susceptibility to *Aeromonas salmonicida* ssp. *salmonicida* challenge. From this study there was no evidence of consistent differences in immmunocompetence or disease susceptibility of diploid or triploid Atlantic salmon.

1. General Introduction

1.1 Introduction

The farming of Atlantic salmon is an important industry in Scotland. However, one of the major problems facing salmon farmers is grilsing; the maturation of fish after only one year at sea. A relatively high percentage of Scottish salmon stocks mature as grilse (20-30%) (Johnstone, 1992; Johnstone *et al.*, 1991). Maturation leads to deteriorative changes in flesh quality with the diversion of energy from flesh growth to the development of reproductive tissue. This requires farmers to sort stocks, identify grilse and sell them at smaller, less profitable sizes. This is time and labour intensive, and sale takes place at a time in the rearing cycle when profit will be reduced. Therefore, grilsing has practical and financial implications for Atlantic salmon farmers.

A control of maturation would allow for the rearing of larger, more profitable fish by eliminating grilsing and this has created interest in the production of sterile fish. Sterile fish can be produced in a variety of ways but one of the most efficient and effective is triploidisation. All-female, triploid salmon are both functionally and hormonally sterile and do not exhibit any of the deleterious effects associated with maturation (Johnstone, *et al.*, 1991).

Although this, the original rationale, is entirely commercial in nature, there has more recently developed an environmental reasoning for the use of triploids in aquaculture. The use of sterile stocks to minimise the perceived threat of interbreeding of escaped farmed salmon and wild indigenous stocks and the associated loss of genetic identity of the indigenous stocks, has also caused triploids to become of interest.

Of the fish examined from 54 Norwegian rivers in 1987, 15 to 20% were escapees (Sattaur, 1989). Escaped farm fish compete with wild fish for food and spawning grounds, and vertical transmission of disease may introduce new diseases into an environment. Potential interactions between wild salmon and escapees from marine salmon cages may also cause changes in the genetic composition of the wild stocks

(Krueger and Kohlmann, 1993; McNab *et al.*, 1987). Interbreeding of escaped farm stocks with wild stocks may result in genetic introgression causing 'dilution' or reduction of genetic variability between populations via the loss of alleles presumed to be adapted to local conditions. Populations evolve to fit the ecological niche they fill, but interbreeding with a non-resident strain may cause the locally adapted native stocks to be replaced by a more homogenous population. However, there is no firm evidence which confirms whether or not there is a genetic risk to natural stocks (Pepper, 1991). Nevertheless, production of non-maturing salmonids in farms would obviate any perceived risks resulting from interbreeding and remove the introgression concern. However, Kruegar and May (1991) suggest that the introduction of an infertile strain may have indirect effects on the native populations. For example by reducing effective breeding sizes if sexually competent animals attempt to breed with those which are incompetent, the overrunning of redds by infertile fish and the competition for resources.

Triploidisation has some major consequences other than sterility on the physiology of a fish and there is an established view that triploid fish do not perform as well as diploids in culture (Benfey, 1997), including that they are more susceptible to stress and disease. This view has made them unpopular for culture. However, there is very little information regarding the impact of triploidisation on the immunocompetence of fish and no published literature on its impact on the immunocompetence of Atlantic salmon. This study aims to compare some defence mechanisms of diploid and triploid Atlantic salmon, to expand the existing knowledge of the immune system of triploid fish, to potentially identify any advantages or disadvantages of triploidy with regards to resistance to disease and to determine whether triploids suffer greater immunosuppression under stress.

1.2 Triploidy in Fish

1.2.1 Introduction

Triploid animals have three sets of chromosomes in every somatic cell rather than the

usual number of two (diploid). Spontaneous triploids are encountered in many species of fish including many salmonids (Arai *et al.*, 1993; Flajshans *et al.*, 1993; Matsubara *et al.*, 1995; Thorgaard and Gall, 1979).Spontaneous triploids in fish probably result from the fertilisation of an unreduced ovum with a normal sperm, by dispermy where one egg is fertilised by two sperm or retention of the second polar body during meiosis II (Arai, *et al.*, 1993; Thorgaard and Gall, 1979).

1.2.2 The Induction of Triploidy

Sterility in fish has been attempted by physical (direct removal of gonads, exposure to gamma irradiation), chemical (hormonal treatment) and biological (induction of immunological interference, induction of triploidy) means (Bye and Lincoln, 1986; Johnstone, *et al.*, 1991). To date only the induction of triploidy has been demonstrated at a commercial level (Pepper, 1991).

Triploids can also be produced by crossing tetraploid animals with diploids (Chourrout *et al.*, 1986) or by the application of physical (temperature shock, pressure shock) or chemical (anaesthetic) treatments to fertilised eggs (Benfey, 1989; Chourrout, 1987; Pepper, 1991; Purdom, 1983; Thorgaard, 1986; Thorgaard and Allen, 1987). Although thermal, chemical and pressure treatments each have a slightly different mode of action, each ultimately results in triploidy by preventing the second meiotic division, thus causing the retention of the "extra" maternal chromosome set (see Figure 1.1)

During normal fertilisation, the spermatozoa (containing one set of chromosomes) enters the egg (containing 1 set of chromosomes and the 2nd polar body) causing the extrusion of the 2nd polar body. This results in a cell containing 2 sets of chromosomes; one paternal and one maternal set. This condition is maintained through all subsequent mitotic divisions (Figure 1.1). During triploidisation, the spermatozoa enters the egg as normal. However, the application of a triploidisation treatment prevents the extrusion of the 2nd polar body. The cells in induced triploids therefore contain two maternal and one paternal chromosome sets. This condition is maintained through all subsequent mitotic divisions (Figure 1.1). Triploid yield (the product of triploid rate and survival

rate) varies with intensity, duration and timing after fertilisation of the treatment (Johnstone, *et al.*, 1991). It has been found that pressure shock produces the best triploid yield for Atlantic salmon (Boulanger, 1991; Johnstone, *et al.*, 1991).

Triploidisation has been carried out in many fish species including rainbow trout, (Happe *et al.*, 1988), Atlantic salmon (Benfey and Sutterlin, 1984; Johnstone, 1996; Johnstone, *et al.*, 1991), brook trout (Boulanger, 1991), brown trout (Brydges and Benfey, 1991), Pacific salmon (Utter *et al.*, 1983), masu salmon (Kitamura *et al.*, 1991), Arctic char [O'Keefe, 1996 #373], tilapia (Koteeswaran *et al.*, 1995), yellow perch (Malison *et al.*, 1993), European catfish (Linhart and Flajshans, 1995), tench (Flajshans *et al.*, 1993), common carp (Recoubratsky *et al.*, 1992), Japanese phytophagous crucian carp (Xinqi *et al.*, 1993), red sea bream (Guxiong, 1993), koi carp (Cherfas *et al.*, 1990), fighting fish (Kavumpurath and Pandian, 1992), loach (Matsubara, *et al.*, 1995), zebrafish (Kavumpurath and Pandian, 1990) and plaice (Lincoln, 1981). Experimental triploidisation of various hybrids has also been investigated (Dorson *et al.*, 1991; Gray *et al.*, 1993; McKay *et al.*, 1992)

1.2.3 The consequences of triploidy

Induced triploidy causes three key differences in a fish in comparison to a diploid. One of these differences is genetic whereas two are physiological and can be related to many of the physiological changes associated with triploidy. These differences are

1. possession of an extra chromosome set
2. disruption of gamete and gonadal development.
3. larger and fewer cells.

The consequences of possession of an extra chromosome set

A chromosome is a unit of hereditary material, which consists of many smaller units called genes. A gene, either by itself or in association with other genes, determines a characteristic of an organism. Genes may exist in different forms; these are called alleles. The genetic combination of alleles is called the genotype. The phenotype is the

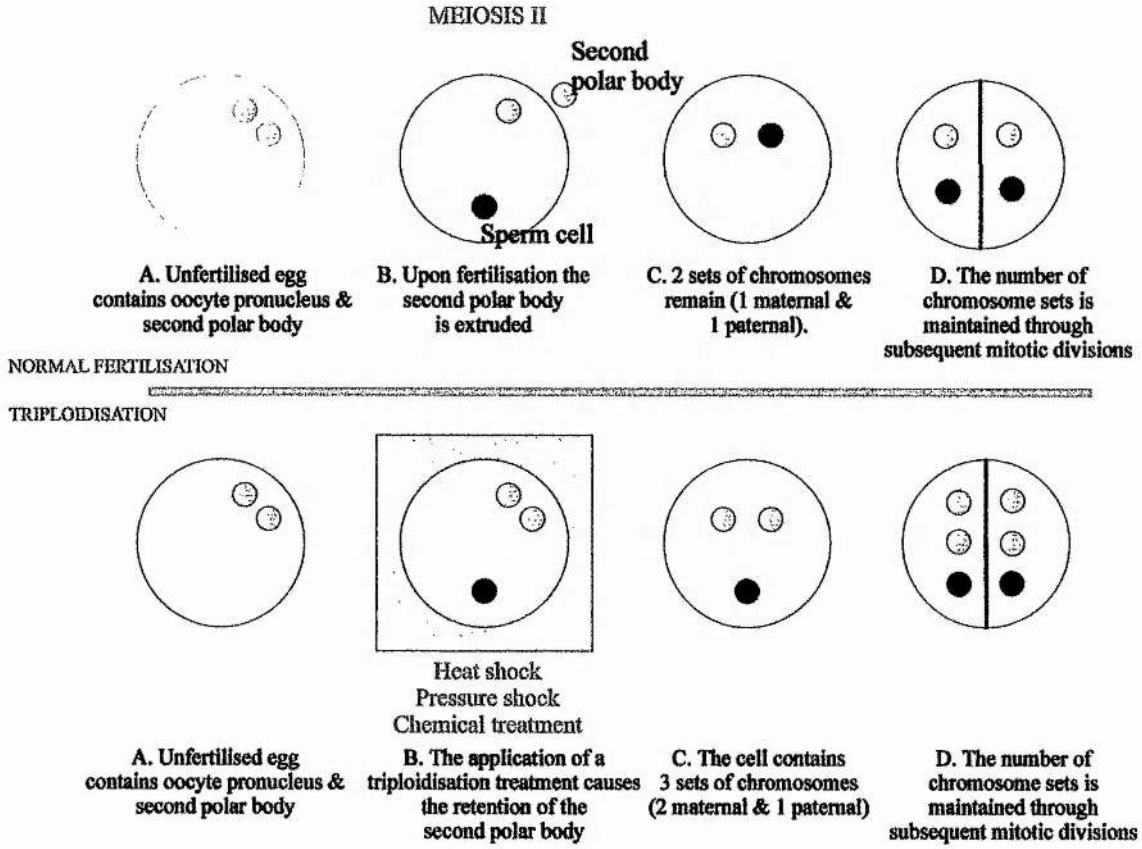


Figure 1.1 The artificial induction of triploidy. The application of a triploidisation treatment shortly after fertilisation causes the retention of the second polar body. This results in the possession of 3 sets of chromosomes (triploidy) rather than 2 (diploidy).

actual appearance of the organism. Phenotypes can result from the influence of many genes and be affected by the interaction between genotype and environment. In a diploid situation there are 2 sets of chromosomes and each will contain an allele for a particular characteristic. Each allele may be either 'dominant' or 'recessive'; a dominant allele is the allele which determines the phenotype of an organism if the organism is heterozygous (ie. has 1 dominant and 1 recessive allele).

In a triploid situation there are 3 sets of chromosomes and therefore 3 alleles for each gene. However, it is not known how this affects the phenotype. There may be 2 theoretical situations:

1. All 3 alleles function. This would result in a greater number of potential outcomes, and therefore more heterozygosity and more individual variation.
2. Only 2 alleles function. The 3rd allele may not exert an influence or only exert an influence under certain (unknown) conditions. It may be that the choice of the 'non-operative' allele is totally random and differs from cell to cell.

The disruption of gamete and gonadal development

Normal gametogenesis is impaired in triploids due to the inability of three homologous chromosomes to distribute correctly and evenly during the first and second meiotic division. As a consequence triploids are often considered sterile. However, there is a difference in the degree of sexual development between the sexes (Benfey, 1991; Benfey and Sutterlin, 1984). Triploidy inhibits the reproductive development of female gonads by completely suppressing oocyte development. Triploid males can mature attaining normal secondary sexual characteristics and developing reduced testes, but their milt is not viable. This difference between the sexes may be due to the number of gametes produced. Females normally produce relatively few oocytes whereas males produce large numbers of spermatozoa, increasing the potential in triploid males for some meiotic divisions to occur normally and thereby enabling a small number of spermatozoa, with unbalanced chromosome numbers, and dilute milt to be produced (Benfey and Sutterlin, 1984). Both males and females are therefore functionally sterile. In triploid females, the inability to produce functional oocytes is probably accompanied

by an altered development of the associated endocrine tissue (Benfey, 1997). In female salmonids those hormones directly associated with reproduction are produced in the thecal and granulosa cells of the ovarian follicular tissue. However, their development is only stimulated after completion of meiosis I. Since triploid oogonia do not develop past meiosis I, follicular development does not occur and the production of reproductive hormones does not take place. Consequently, triploid females do not exhibit secondary sexual characteristics or the deteriorative changes associated with maturation.

In male salmonids, the interlobal and interstitial cells of the testes are the sites of production of reproductive hormones. However, the development of these tissues and therefore the production of these hormones is not dependent on the completion of meiosis I. Therefore, triploid males have endocrine profiles similar to those of diploids (Benfey, 1997).

The consequences of triploidy on the cellular physiology of fish

A triploid cell contains 50% more DNA than a comparable diploid cell, due to the presence of an extra chromosome set. Nuclear and cellular size increase in triploid animals to accommodate the increased amount of nuclear DNA, maintaining a normal ratio of nuclear to cytoplasmic material. However, to maintain normal organ and body size, the number of cells is reduced. Therefore, with increased cell size and decreased cell number in a given tissue that remains the same size, the total cell surface area of that tissue or organ must decrease (Benfey, 1991). This increase in cell size has been reported in various tissues, including erythrocytes, neutrophils (Small and Benfey, 1987), brain cells (Aliah *et al.*, 1990; Lou and Purdom, 1984; Small and Benfey, 1987), epithelium (Lou and Purdom, 1984), cartilage (Lou and Purdom, 1984), muscle (Greenlee *et al.*, 1995), kidney (Lou and Purdom, 1984), retinal cells (Small and Benfey, 1987) and gametes and gonads (Chourrout, *et al.*, 1986; Lincoln, 1981) .The difference in the size of erythrocytes of diploid and triploid fish of many species is well documented with a significant difference being found in the median volume of erythrocytes (Benfey and Sutterlin, 1984; Benfey *et al.*, 1984). This difference has been used as the basis of determining ploidy in some studies.

There are two fundamental consequences of increased nuclear and cellular volume in triploids; decreasing surface area and larger intracellular distances. The corresponding decrease in the ratio of surface area to volume could affect processes limited by surface area. For example, this may affect the binding of hormones or other messenger substances to membranes and possibly metabolite, nutrient and ion exchange, both passively and actively. The increase in cell size and internal volume may cause internal transport and diffusion distances to be increased but would depend on the shape of the nucleus and cell. This may affect processes such as signal transduction from cell surface to the nucleus and possibly the resulting production and transfer of RNA and other proteins.

1.3 The Immune System of Fish

1.3.1 Introduction

The immune system of fish, as in all vertebrates, can be divided into two functional categories; the non-specific immune system and the specific immune system (also known as natural/ innate and adaptive immunity respectively). Non-specific immune processes provide the first line of defence against pathogens in the form of barriers and then by using a variety of cellular and non-cellular responses. The specific immune system also has a variety of cellular and non-cellular parameters, but these provide a response that is specific to each pathogen. Furthermore, specific immunological memory provides protection in the event of re-infection by a pathogen. The specific or adaptive immune system is characteristic of vertebrates (above agnathan fish) (Warr, 1997) and has a capacity for specificity and memory.

1.3.2 Non-specific defence barriers

Non-specific defence barriers include the physical epithelial shield of the scales, skin and mucus. Scales and skin form physical barriers against pathogen penetration and these plus the gut are covered by mucus which helps to prevent attachment to the epithelium. A pathogen may become trapped within the mucus and be sloughed from

the surface or digested by mucosal lytic enzymes. Fish mucus has been found to contain complement, lysozyme, lectins, proteases and immunoglobulins (Alexander and Ingram, 1992; Yano, 1996).

1.3.3 Humoral immune defence mechanisms

Non-specific

Non-specific, humoral defence mechanisms include microbial growth inhibitory substances, enzyme inhibitors, lysozyme, complement, agglutinins and precipitins and other substances less well described in fish (Yano, 1996).

Microbial growth inhibitory substances include a variety of proteins which deprive bacteria of nutrients and interferons. Proteins such as transferrin, caeruloplasmin and metallothionein, chelate metal ions and therefore deprive bacteria and other parasites of essential inorganic ions (Alexander and Ingram, 1992; Roed *et al.*, 1995). Transferrin is an iron binding protein and is one element of the mechanism termed iron with-holding (Weinberg, 1984; Weinberg, 1992). This system attempts to deny invading organisms and neoplastic cells of iron whilst permitting normal host cells access. This mechanism, in mammals, is complex and includes defensive and inducible strategies. Defensive strategies place iron binding proteins in areas of potential pathogenic invasion such as eggs, plasma, saliva (Weinberg, 1984). Inducible responses lower intestinal assimilation of iron, cause retention of iron by macrophages, synthesis of ferritin (an iron storage protein), mobilisation of neutrophils to areas of infection or injury, lowering of plasma iron levels and the removal of iron to the liver (Otto *et al.*, 1992; Weinberg, 1984; Weinberg, 1992). This system is not well described in fish but it is known that fish possess serum transferrin (Hershberger and Pratschner, 1981; Roed, *et al.*, 1995; Utter *et al.*, 1970).

Interferons (IFN's) are mainly anti-viral proteins or glycoproteins that interfere with viral replication (Alexander and Ingram, 1992; Yano, 1996). IFN production has been confirmed in teleosts (Yano, 1996) and IFN-αβ has been shown to be produced by fish cells, *in vivo* and *in vitro*, in response to viral infection (de Kinkelin *et al.*, 1982; Dorson

et al., 1992; Yano, 1996). Also, leucocytes from rainbow trout kidney were shown to secrete IFN-γ like molecules in response to mitogen stimulation (Graham and Secombes, 1990).

Enzyme inhibitors inhibit the function of otherwise potentially hazardous enzymes which may be secreted by an invading organism. Those so far detected in fish appear to be principally antibacterial. These are mainly antiproteases such as α_1-proteinase inhibitor (previously known as α_1-antitrypsin) and α_2 macroglobulin (Alexander and Ingram, 1992; Ellis *et al.*, 1981; Yano, 1996). α_2-macroglobulin (α_2M) activity has been determined in several fish species (Bowden *et al.*, 1997).

N-acetylmuramide glycanohydralase better known as lysozyme or muramidase, is a lysin that attacks structures within the cell walls of bacteria. Lysozyme has a direct anti-microbial effect but, in mammals, it has also been shown to promote phagocytosis by opsonisation of bacteria or by direct activation of phagocytes (Yano, 1996). It has been identified and studied in several fish species, and is distributed mainly in tissues rich in leucocytes (blood, kidney, spleen) or in areas of potential bacterial invasion (skin, gills, gut) (Grinde, 1989; Holloway *et al.*, 1993; Lie *et al.*, 1989; Ourth, 1980; Yano, 1996). Lysozyme is present in neutrophils and it has been suggested that these may be an important source of lysozyme in plasma or serum (Fletcher and White, 1973). Two lysozyme variants (types I and II) have been identified from the kidney of rainbow trout (Grinde, 1989). Type I was found to be a potent antimicrobial agent and results suggest that unlike mammalian lysozyme, fish lysozyme has antimicrobial activity against Gram-negative as well as Gram-positive bacteria (Yano, 1996), even in the absence of complement. Lysozyme from eggs, serum and skin mucus of fish has also been shown to have antimicrobial properties (Yano, 1996).

The complement system is composed of two distinct pathways; the classical (antibody dependent) and the alternative (antibody independent). Complement is a group of serum proteins and stimulation of the complement system triggers sequential biochemical reactions. Teleost fish have the alternative and classical complement pathways

comparable to the mammalian complement system. The classical pathway is initiated by the antibody-antigen reaction, which leads to the activation of the first component C1. The initiation of the alternative pathway in mammals is by different mechanisms, including bacterial endotoxins, fungal polysaccharides, plant factors and animal venom (Sakai, 1992). These activate C3 (Whaley and Lemercier, 1993). The activity of serum complement maintains a defined constant level in healthy individuals, but can fluctuate in response to physiological and pathological changes. Complement is not produced as a response to antigenic stimulation but rather acts in conjunction with antibody and phagocytic cells to provide and enhance specific effector functions (Sakai, 1992).

Agglutination and precipitation can be caused by the same substance if the molecule is large enough. Agglutination refers to the aggregation of cells whereas precipitation refers to the aggregation of molecules. These substances are classified into two groups; immunoglobulins (Igs) (although only IgM has been identified in fish) and lectins (Alexander and Ingram, 1992). Agglutinins, which react with sugar residues on erythrocyte or bacterial cell walls, are usually lectins or lectin-like molecules. Both C-type (calcium dependent) and S-type (thiol dependent) lectins are present in fish as with mammals. In fish they are present in eggs, serum and skin mucus (Yano, 1996) and appear to play an antibacterial and antifungal role (Alexander and Ingram, 1992; Yano, 1996). A mannan-binding protein (MBP) has been isolated from the serum of Japanese eel and Atlantic salmon. In mammals this protein acts as an opsonin and activates complement. Although lectins in fish have been shown to provide protection against some diseases and can inhibit growth of virulent strains of bacteria (Yano, 1996), the mechanisms of this protection are not well understood.

Natural, non-Ig precipitins, for example α-precipitin and C-reactive protein (CRP), have been described in several fish species (Alexander and Ingram, 1992; Yano, 1996). It has been suggested that CRP, which can recognise phosphorylcholine, a major component of bacterial, fungal and parasite cell walls, may play a vital role in the immune system of fish as a surrogate immunoglobulin, since fish produce only one antibody isotype (IgM). CRP also activates the complement system (Yano, 1996).

Other substances that may have roles within the non-specific, humoral immune system of fish include proteinase and non-specific lysins. Proteinases within the mucus of fish are capable of lysing Gram-negative bacteria upon their contact with the skin surface (Hjelmeland *et al.*, 1983). Non-specific lysins against erythrocytes, bacteria and other cellular antigens are found in serum, eggs and skin mucus. These lysins are natural peptides which act spontaneously without interaction with complement or antibody (Alexander and Ingram, 1992).

Specific

B-like lymphocytes produce and secrete antibodies. Antibodies are known collectively as immunoglobulins (Ig). Fish produce only one antibody isotype; IgM (Du Pasquier, 1982). This is in contrast to mammals which have the ability to produce a large range of isotypes.

Antibody can combat some pathogenic antigens simply by binding to them. This can be a direct blocking of a critical function of an antigen (for example blocking receptors of host cells or active sites of enzyme) (Kaattari and Piganelli, 1996). Also, the binding of antibody molecules and antigens can result in macromolecular complexes which can be sufficiently large that the complexes will precipitate. A similar reaction can occur at a cellular level causing the agglutination of cells (Kaattari and Piganelli, 1996).

However, antibody probably plays a more important role by activating the classical complement pathway and/or acting as an opsonin thereby promoting phagocytosis. Opsonisation is a process by which antibody (or sometimes other immunological factors such as complement, lectins or CRP) promotes phagocytosis of an antigen. In basic terms, opsonisation is the coating of antigenic particles with a substance that is more easily recognised by phagocytes. Activation of the complement system is an important immunological defence mechanism and its consequences are described Chapter 3.1.4.

1.3.4 Cells of the immune system

Phagocytes

Although phagocytes have a non-specific function, they also have a fundamental role in the specific immune response.

Phagocytic cells in fish are granulocytes (neutrophils), macrophages and monocytes although the only granulocytes present in some species are neutrophils (Secombes and Fletcher, 1992). Phagocytes are often referred to as mononuclear phagocytes (macrophages and monocytes) and polymorphonuclear leucocytes (granulocytes). However, granulocytes in some fish species are not polymorphonuclear.

Neutrophils are present in large numbers in blood and haemopoietic organs. In normal conditions they are rare in tissues (Afonso, *et al.*, 1998). Macrophages are present in many tissues and body cavities, and descend from blood monocytes which can migrate into tissues or areas of inflammation as required and mature into macrophages (Afonso, *et al.*, 1998). Their responsiveness is non-specific and they can be activated non-specifically to increase their phagocytic and killing ability. However, they are also affected by cells and humoral factors of the specific immune response (eg. Macrophage Activating Factor (Secombes, 1996)).

Macrophages are often the first phagocytes to encounter invading organisms due to their distribution throughout the fish host (Afonso, *et al.*, 1998). Phagocytic cells are chemically attracted to the site of infection by chemoattractants derived from both pathogen and host, and attempt to kill the pathogens (Ainsworth, 1992; Secombes and Fletcher, 1992). As in mammals, this cellular response is biphasic. Neutrophils respond quickly and arrive in the area of inflammation before the additional macrophages (Afonso, *et al.*, 1998). The accumulation of neutrophils occurs within an hour of giving an inflammatory stimulus and peaks after 48 hours (Afonso, *et al.*, 1998).

Neutrophils and macrophages work together and the transfer of neutrophilic material to macrophages during inflammation has been observed (Afonso, *et al.*, 1998). It has

also been shown that fish macrophages may form multinucleate giant cells, which may occur during the chronic inflammatory response, and these giant cells have a phagocytic ability (Secombes, 1985). There are several mechanisms responsible for removing or killing pathogens including phagocytosis, production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), secretion of cytokines and eicosanoids (Ainsworth, 1992; Chung and Secombes, 1988; Secombes and Fletcher, 1992). These are described in more detail in Chapter 6.1.

Fish neutrophils have been reported to exhibit only slight phagocytic capacity, in comparison to mammalian neutrophils (Sakai, 1984). However, this may depend on the host species and/or pathogen. Ainsworth (1992) has convincingly shown phagocytosis of *Aeromonas hydrophilia* by catfish neutrophils. In contrast, Lamas and Ellis (1994) found that Atlantic salmon blood neutrophils exhibit a strong respiratory burst but little phagocytosis in response to *A. salmonicida*. Ellis (1981) postulated that the main role for neutrophils may be a bacteriocidal effect which is extracellular. However, Griffin (1983) suggested that neutrophils may require a stimulus to provoke their full phagocytic ability, with this stimulus as yet being unknown.

Lymphocytes

The cells within the specific immune system, which play a dominant role are lymphocytes. There are two main lymphocyte types present in fish: T-like (thymic derived) and B-like (develop independently of the thymus) cells (Manning and Nakanishi, 1996). T-like lymphocytes recognise fragments of antigen, by using T cell receptors (TCR), bound to molecules encoded in the major histocompatability complex (MHC). A systematic nomenclature of T cells has been determined for mammals (the 'cluster of differentiation' (CD) system), which has allowed the definition of cell subsets according to the molecules expressed on the cell surface.

Similar information is not available in fish. However, using monoclonal antibodies (MAb's) to serum Ig, a subpopulation of lymphocytes have been shown to be surface Ig positive (SIg+). These are B-like lymphocytes and can be purified using MAb's to

the SIg marker. There are, as yet, no MAb's to specifically purify T-like lymphocytes. However, T cell receptor (TCR) genes have been identified in some fish species (Partula *et al.*, 1996; Wilson *et al.*, 1998) which may lead to the production of MAb's to recombinant proteins expressed by the TCR gene.

Lymphocytes are present in blood and major lymphoid organs of fish. There is also evidence that integumental tissues such as skin, gills and gut have lymphocyte populations (Davidson *et al.*, 1997; Lin *et al.*, 1999). Lymphocytes are attracted to areas of inflammation as well as phagocytes. Finn and Nielson (1971) believed these to be 'background' cells with no function in the site of inflammation. However, Ellis (1986) reviewed evidence that, similarly to mammals, lymphocytes in fish release a variety of lymphokines which play a central role in the attraction and activation of circulating cells and other inflammatory responses.

T-like lymphocytes are responsible for cell-mediated specific immune responses whereas B-like lymphocytes are responsible for specific, humoral immunity and transform into plasma cells which synthesise immunoglobulin (Bognor and Ellis, 1977; Manning and Nakanishi, 1996). Each lymphocyte (whether B-like or T-like) is capable of recognising only one particular antigen. When an antigen binds to the few cells that can recognise it, this induces lymphocyte proliferation. This results in an antigen selecting for the specific clones of its own antigen-binding cells, a process known as clonal selection (Manning and Nakanishi, 1996; Roitt *et al.*, 1993).

T-like lymphocytes have a variety of functions mediated by different T-cell subsets. Using T cell receptors (TCR's), T-like lymphocytes can recognise fragments of antigen bound to molecules encoded within the major histocompatibilty complex (MHC). The MHC molecules that present processed antigens to T cells occur on most cells but antigen presenting cells are specialised. These are described below. The functions of fish T-like lymphocytes are thought to be very similar to mammalian T cells, including direct killing of target cells and co-operation with B-like cells to increase antibody and cytokine production (Manning and Nakanishi, 1996; Warr, 1997).

Cytotoxic cells

Non-specific cytotoxic cells are considered to be equivalent to mammalian natural killer cells (Evans and Jaso-Friedmann, 1992). They are present mainly in kidney and the peritoneal cavity, with only a small number in circulation. These cells have the ability to lyse target cells on cell to cell contact.

Antigen Presenting Cells

Antigen presenting cells (APC's) in fish probably act in a similar fashion to mammalian APC's. (Manning and Nakanishi, 1996; Warr, 1997). In mammals, a wide spectrum of cells can present antigen depending on where and how the antigen first encounters the cells of the immune system. Dendritic cells, macrophages, monocytes, B cells and some T cells can all act as APC's; each may most efficiently activate certain T cell populations eg. interdigitating dendritic cells are considered most effective for activation of resting CD4+ T cells in mammals (Roitt, *et al.*, 1993).

Eosinophilic granular cells

Eosinophilic granular cells (EGC's) are considered to be the piscine equivalent to mast cells (Ellis, *et al.*, 1981). They are present in large numbers of the intestinal tract. Similar cells occur in skin, gills and swim bladder (Reite, 1997). These cells react to injury or infection by massive degranulation (Reite, 1997). This degranulation includes the release of vasoactive compounds (Reite, 1997)which in turn can initiate the inflammatory response.

1.4 Triploidy and disease resistance

1.4.1 The effect of triploidy on the immune system of fish

One of the major differences between diploid and triploid fish is cellular size. Triploid cells are one third larger than diploid cells, due to the increase in genetic material. There are two fundamental consequences of increased nuclear and cellular volume in triploids; decreasing ratio of cell surface area to volume and larger intracellular distances. The decrease in the former could affect processes limited by surface area.

The increase in cell size and internal volume may theoretically cause internal transport and diffusion distances to be increased. However, this would depend on the shape of the nucleus and cell.

The possible differences in extra- and intra-cellular dynamics between diploid and triploid fish, may also have consequences for immunocompetence and disease resistance. For example, do these extra- and intra-cellular differences cause any differences in protein production and distribution? Are the humoral factors of both the non-specific and specific immune system produced to the same quantity as for diploids and are they produced and distributed as quickly? Do the cellular components migrate at the same rate in triploids as in diploids? If there are a third fewer cells but they are a third larger, how does the total efficiency of a triploid cellular immune system compare to that of a triploid? Triploid rainbow trout neutrophils and triploid ayu kidney leucocytes have been reported to have increased phagocytic activity (Kusuda *et al.*, 1991; Yamamoto and Iida, 1995b). Complement and bactericidal activity is no greater in triploid rainbow trout or ayu than it is for diploids (Kusuda, *et al.*, 1991; Yamamoto and Iida, 1995a, b). Agglutinating antibody titres of vaccinated diploid and triploid ayu did not differ (Kusuda, *et al.*, 1991). The only difference which did occur was that bactericidal activity of triploid rainbow trout serum remained constant whilst that of diploids decreased through the spawning period. It was suggested that this may explain the higher survival of triploid females during the post-spawning period compared with diploids (Yamamoto and Iida, 1995a). However, the immune system is immensely complicated and is composed of many factors. A more thorough investigation of the triploid immune system is necessary in order to draw more definitive conclusions.

1.4.2 The disease susceptibility of triploid fish

The comparative susceptibility of triploid and diploid fish to disease is still debated. Challenges with bacterial kidney disease (BKD) in New Brunswick have indicated that triploid Atlantic salmon have a lower survival rate and die sooner than diploid controls (Anon., 1994). Myers and Hershberger (1991) also noticed that triploid rainbow trout were generally more susceptible to *Columnaris* and *Ichthyophthirius* spp. than their

diploid conterparts. However, many challenges have found no consistent differences in the susceptibility of triploid and diploid fish to disease, for example, triploid ayu to *Vibrio anguillarum* (Inada *et al.*, 1990) and rainbow trout to infectious haematopoietic necrosis (IHN), furunculosis and vibriosis (Yamamoto and Iida, 1995c). Bruno and Johnstone (1990) also found no consistent differences between diploid and triploid Atlantic salmon when challenged with BKD but in one trial where a difference did occur, triploids were more resistant.

No differences have been found in responses to vaccination (Inada, *et al.*, 1990; Kusuda, *et al.*, 1991; Yamamoto and Iida, 1995c).

1.5 Project structure and aims

1.5.1 Triploidisation

Atlantic salmon , *Salmo salar* were triploidised using hydrostatic pressure shock as described by Johnstone (1992). Briefly, this involved fertilised eggs being exposed to 10,000 PSI for 5 min in a 2L pressure vessel at 30 min post-fertilisation. For some of the experiments in this study, all-female stocks were used. Therefore, the milt used for fertilisation of the eggs was taken from a sex-reversed female. The process of sex-reversal is described by Johnstone (1992). Triploidisation and sex-reversal were carried out by Philip MacLachlan, and most fish were reared at the Fish Cultivation Unit, Aultbea.

1.5.2 Ploidy confirmation

Many studies have shown that the measurement of the major axis of either the cell or nucleus of peripheral blood erythrocytes is a reliable method for determining ploidy. A study by Benfey *et al.*, (1984) showed that in Atlantic salmon, the measurement of this axis alone was as reliable for confirming ploidy as the calculation of cell surface area or nuclear volume which requires the measurement of more than one axis. Benfey *et al*, (1984) also showed that the measurement of erythrocyte cell or nucleus major axis from blood smears is as accurate for identifying triploids as using a coulter counter or flow

cytometer. Therefore, ploidy was usually confirmed for every individual from examination of blood smears.

Examination of gonads was also used when all-female fish were being destructively sampled. Figure 1.2 shows examples of gonads from diploid and triploid female fish

1.5.3 Aims

The aim of this thesis is to compare a variety of immune parameters of diploid and triploid Atlantic salmon in order to obtain an overview of the mechanisms of the triploid immune system.

Chapter 2 presents data which confirms that the leucocytes within peripheral blood of Atlantic salmon are larger in triploids, and how much larger they were. Although, the comparative sizes of erythrocytes and other cell types of several species of diploid and triploid fish have been described, there is only one report of leucocyte sizes in the literature. This report by Small and Benfey (1987) compared the sizes of polymorphonuclear leucocytes from diploid and triploid Atlantic salmon. No other leucocyte types have been compared in the literature.

Chapters 3 and 6 report results from investigations attempting to establish normal, baseline differences between diploids and triploids in non-specific, humoral and cellular immune parameters. Work presented in Chapter 4 extended the investigation by examining the kinetics of the non-specific humoral response in response to an endotoxin.

Chapter 5 comprises a study designed to establish whether there may be an iron binding protein in peripheral blood leucocytes. Results from this study help to explain how the immune system of salmon is reacting to the endotoxin administered in Chapter 4.

In order to compare the specific responses of diploids and triploids an antibody dynamics trial was carried out. This monitored the antibody response of diploids and

triploids to a commercial vaccine. This experiment is described in Chapter 7.

In response to results generated within this comparison of diploids and triploids, an additional experiment was carried out (Chapter 8) to compare immune parameters in diploids and triploids from different families.

Finally, the experiment outlined in Chapter 9 was an attempt to determine why triploid fish seem to be more susceptible to disease in comparison to diploids in farming conditions, but not in laboratory trials. It was hypothesised that exposure to chronic stress may lower the immunocompetence of triploids in comparison to diploids, and subsequent disease challenge would result in an increased disease susceptibility of the triploid fish.

Chapter 10 is a general discussion which aims to bring the diverse results from this thesis together.

A. **B.**

Figure 1.2 Ovarian squashes of gonads taken from triploid (a) and diploid (b) female, Atlantic salmon. Diploid fish have ovaries which are orange in colour with developing oocytes and melanin spots evident. Triploid fish have ovaries which are comparatively grey in colour with no developing oocytes but the lamellar structure of the ovigerous folds is clearly visible.

2. The characterisation of some haematological parameters in diploid and triploid Atlantic salmon

2.1 Introduction

In amphibians, fish and shellfish, the increase in nuclear material caused by triploidisation results in both increased nuclear volume and cellular volume, but cell numbers are reduced to maintain organ size to normal ranges (Allen, 1983; Fankhauser *et al.*, 1942; Swarup, 1959). The decrease in cell numbers in triploid animals appears to be a homeostatic mechanism that acts to maintain standard organ size. This mechanism is operational in animals but not in plants. Triploidy in plants results in giantism (Swarup, 1959). The nature of this mechanism is not known but it is interesting to note that it operates not only for fixed organs such as kidney, spleen etc, but also in the blood. Erythrocytes of triploid fish of several fish species, including salmonids, have been shown to be larger than those of comparable diploids (Benfey, 1997; Benfey *et al.*, 1984; Small and Benfey, 1987) and fewer in number (Small and Benfey, 1987). The balance of reduced cell number and increased cell size results in the haematocrits of triploid fish being comparable to those of diploids (Benfey, 1997; Yamamoto and Iida, 1994).

However, there is only one report on the increased size of peripheral blood leucocytes (Small and Benfey, 1987). This reports that leucocytes were uniformly larger in triploid Coho salmon, *Onchorynchus kisutch*, and Atlantic salmon, *Salmo salar*, in comparison to diploids. The leucocytes measured were polymorphonuclear leucocytes (peripheral blood granulocytes). To the authors knowledge there are no reports on the comparative sizes of any other leucocytes in the literature.

There are two fundamental consequences of increased nuclear and cellular volume in triploids;

- *decreased surface area to volume ratio.* Literature suggests that increased cell length and width in triploid cells, is not accompanied by a decrease in cell

height (Benfey and Sutterlin, 1984; Sezaki *et al.*, 1991). Therefore, triploid cells should have a lower surface area: volume ratio and any function limited by surface area should be consequently reduced in triploids. However, the conclusion of unchanging cell height should be considered with care since this parameter was not measured directly, but rather derived. There are a variety of problems associated with measuring cell dimensions (such as cell shrinkage during fixation) which may mean that these conclusions do not accurately portray the *in vivo* situation. In addition, it is interesting to note that studies of polyploid cell populations have shown that those cells which possess nuclei with highest ploidy also have highly indented cell membranes (Brodsky and Uryvaeva, 1978), and therefore the assumption of decreased surface area to volume ratio in triploid cells may be inaccurate.

- *larger intracellular distances.* The increase in cell size and internal volume may theoretically cause internal transport and diffusion distances to be increased. However, this would depend on the shape of the nucleus and cell, and it has been reported that the shape of triploid cells differs from that of diploid, with triploid cells being larger mainly as a result of increased length (Benfey, 1997).

This chapter aims to confirm that the differences stated in the literature for haematocrit and erythrocyte numbers of diploid and triploid fish, are also true for Atlantic salmon. Also, it is often assumed that leucocyte numbers and sizes follow the pattern of erythrocytes and other cells, in that triploid cells are larger and fewer in number. This chapter aims to affirm or deny this assumption for leucocytes in peripheral blood.

2.2 Materials and methods

2.2.1 Fish

Packed cell volume

Packed cell volumes were determined on samples collected during July 1995 (water temperature 12.4 °C), February 1996 (5.9 °C) and June 1996 (12.2 °C). Sibling diploid and triploid, all-female Atlantic salmon were reared and maintained at the Fish Cultivation Unit, Aultbea, in 1 m^3 tanks with a flow-through sea water supply. The average weight was approx. 1.2 kg, 800g and 1 kg for the July 1995, February 1996 and June 1996 populations respectively.

Total cell counts

Sibling diploid and triploid, all-female Atlantic salmon were reared and maintained at the Fish Cultivation Unit, Aultbea. The fish were maintained at ambient temperature (mean 10°C) during the course of the experiment in 1 m^3 tanks with a flow-through sea water supply. The average weight was 37 g.

Differential leucocyte counts and leucocyte measurements

Blood smears were collected from sibling diploid and triploid Atlantic salmon from three families of the same age (one sea winter). These fish were reared and maintained at Matre Havforskningsinstitut, Norway. Water temperature was approx. 12.5 °C at the time of sampling. These families were designated a code to describe the individual mother and father. The letter was attributed to the father and the number to the mother. The families were therefore coded as follows: J20; O18; I18. Families J20 and I18 were mixed sex with milt being derived from a normal male. Family O18 was an all-female population with the milt being derived from a sex-reversed female. Therefore families O18 and I18 were half-siblings. The average weight of the sampled fish was 89.1g, 87g and 82.3g for families J20, O18 and I18 respectively. These fish were also assayed for non-specific, humoral parameters. Those assays are described and discussed in Chapter 8.

2.2.2 Sample collection

Packed cell volume

The fish were anaesthetised by immersion in tricaine methanesulfonate (MS-222, Sigma) at a final concentration of 2mg l^{-1} and peripheral blood sampled from the caudal vein using heparinised vacuettes (Greiner) and needles. Blood was collected from the vacuettes into sodium heparinised capillary tubes. Samples were collected in duplicate for each fish when possible. Tubes were then sealed with Cristaseal and centrifuged at 19872 g for 5 min in a haematocrit centrifuge. The packed cell volume was determined using a sliding scale and expressed as a percentage of the blood volume.

Formalised blood for total cell counts

Peripheral blood samples were collected from the caudal vein of anaesthetised fish using heparinised vacuettes (Greiner) and needles. Blood was diluted 1:10 with formal saline (0.9% NaCl + 10% formalin). The formalised blood was further diluted in 0.01M phosphate buffered saline (PBS) to a final dilution of 1 in 100, and total cell counts made using a haemocytometer.

Blood smears for differential cell counts and leucocyte measurements

A small drop of blood was smeared onto a methanol-cleaned glass slide and allowed to air dry. Duplicate samples were taken from each fish when possible. Once dry, the blood smears were fixed as soon as possible by immersion into 100% methanol for 10 min. These were then stained using a Hema Gurr staining kit (Merck). Differential leucocyte counts and leucocyte sizes were determined from these preparations. Leucocytes were identifed as described in Chapter 2.2.3. When measuring leucocyte sizes 10 slides from each group were examined. On each slide all leucocytes were examined until 10 of the least common leucocytes (monocytes) had been measured.

2.2.3 Cell identification from blood smears

Stained blood smears were examined by light microscopy using x100 oil immersion. Cells were identified as lymphocytes, granulocytes, monocytes or thrombocytes (ovoid, spiked or spindle). Examples of these cell types are shown in Figure 2.2.

2.2.4 Statistical analysis

Percentage data were transformed for statistical analyses using an arcsine transformation. The arcsine transformed data of packed cell volumes and differential leucocyte counts were analysed by one way and two way ANOVA, respectively, using GENSTAT.

Cell count data were analysed by one way ANOVA, and leucocyte sizes by two way ANOVA using GENSTAT.

2.3 Results

2.3.1 Packed cell volume

Means (and standard errors) are shown in Table 2.1.

Table 2.1 Means, standard errors and numbers of observations for the packed cell volume (%) of 3 groups of diploid and triploid, all-female, Atlantic salmon.

	July 1995		Feb 1996		June 1996	
	Diploid	**Triploid**	**Diploid**	**Triploid**	**Diploid**	**Triploid**
Mean	45.95	47.57	57.23	45.19	48.71	43.25
Std Err	0.7	1.7	1.1	1.9	1.8	1.3
No. Obs	19	20	22	16	7	8

There were no statistically significant differences between the diploid and triploid fish at the July 1995 and June 1996 sampling times. However, there is a significant difference at the February 1996 sampling time ($p<0.001$).

The frequency distributions of diploid and triploid packed cell volumes for each sampling time are shown in Figure 2.1. There are no consistent patterns of distribution for the diploids or triploids (ie. The triploids do not have the highest and lowest packed cell volumes consistently).

2.3.2 Cell counts

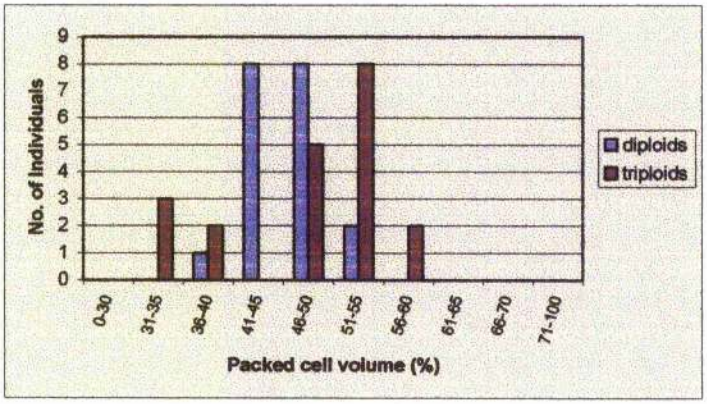
Means, standard errors and the ratio diploids: triploids are shown in Table 2.2, for total blood cell numbers, blood erythrocyte numbers and blood leucocyte numbers.

Table 2.2 Means, standard errors, numbers of observations and diploid/triploid ratio for the numbers of total blood cells, blood erythrocytes and blood leucocytes of diploid and triploid, all-female, Atlantic salmon.

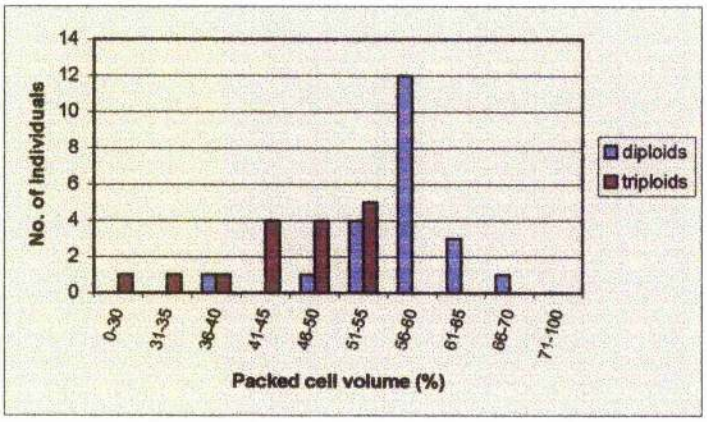
	Total Blood Cell N°s ($x10^8$ cells/ml)			**Blood Erythrocyte N°s ($x10^8$ cells/ml)**			**Blood Leucocyte N°s ($x10^6$ cells/ml)**		
	Diploid	Triploid	**Ratio**	Diploid	Triploid	**Ratio**	Diploid	Triploid	**Ratio**
Mean	1.36	0.72	**1.88**	1.30	0.70	**1.85**	5.87	3.47	**1.69**
Std. Err.	0.16	0.07		0.19	0.06		0.73	0.26	
No. Obs	10	10		7	4		7	4	

There are statistically significant differences between diploid and triploid fish in the total number of blood cells/ml ($p=0.002$) and in the numbers of blood erythrocytes ($p=0.004$) and leucocytes ($p<0.001$), with triploids having less cells. However, the triploid cell counts are not lower by the factor of one third. The total blood cell, blood erythrocyte and blood leucocyte counts of the triploids are slightly more than half of the diploid counts.

A. Packed cell volumes (%) from July 1995



B. Packed cell volumes (%) from February 1996



C. Packed cell volumes (%) from June 1996.

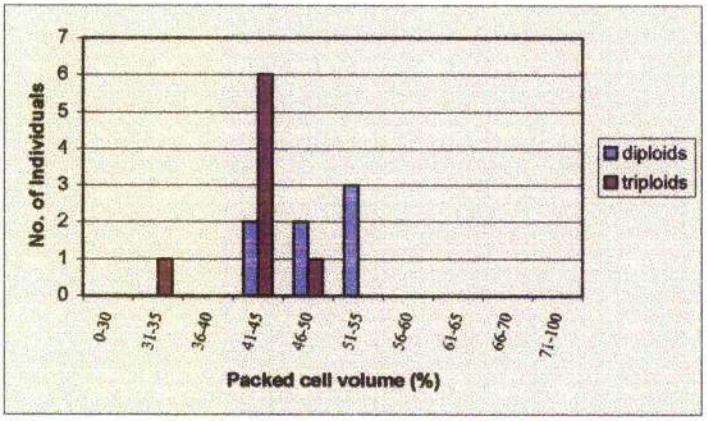


Figure 2.1 Frequency distributions of packed cell volumes (%) of diploid and triploid, all-female Atlantic salmon sampled July 1995, February 1996 and June 1996.

2.3.3 Differential leucocyte counts and sizes

Means and standard errors of differential leucocyte counts are shown in Table 2.3. There are no statistical differences in the percentage leucocyte populations of diploids and triploids within families or between families. Figure 2.2 shows some peripheral blood leucocytes of diploid and triploid salmon.

Table 2.4 shows mean sizes (μm) and standard errors of peripheral blood leucocytes from diploid and triploid Atlantic salmon. There are significant differences in the sizes of all leucocytes present in peripheral blood of diploid and triploid salmon. There are no significant family differences. Table 2.5 shows the diploid/triploid ratio of peripheral blood leucocyte sizes. Triploid cells are larger than diploids. However, the diploid cells are not two-thirds the size of triploid cells (a ratio of 0.66).

Table 2.3 Means, standard errors (in brackets) and numbers of observations for the percentage populations (%) of peripheral blood leucocytes for diploid and triploid, all-female, Atlantic salmon from 3 family groups.

	Family J20 mixed sex		**Family O18 all-female**		**Family I18 Mixed sex**	
	Diploid n=30	**Triploid n=30**	**Diploid n=15**	**Triploid n=15**	**Diploid n=30**	**Triploid n=30**
Granulocytes (%)	4.5 (0.21)	4.8 (0.31)	5.2 (0.32)	4.2 (0.26)	4.2 (0.29)	4.8 (0.25)
Monocytes (%)	0.46 (0.08)	0.62 (0.23)	0.62 (0.13)	0.96 (0.23)	0.47 (0.10)	0.74 (0.11)
Lymphocytes (%)	46.08 (0.69)	42.77 (0.56)	57.31 (1.10)	58.04 (0.81)	55.52 (1.03)	53.36 (0.44)
Thrombocytes (%)	49.12 (0.71)	51.81 (0.53)	36.82 (1.16)	36.75 (0.98)	39.77 (1.06)	39.98 (1.30)

Table 2.4 Means and standard errors (in brackets) of peripheral blood leucocyte sizes (μm) in diploid and triploid Atlantic salmon from 3 family groups.

	Family J20 mixed sex		**Family O18 all-female**		**Family I18 Mixed sex**	
	Diploid	**Triploid**	**Diploid**	**Triploid**	**Diploid**	**Triploid**
Granulocytes	11.2 (0.1)	13.6 (0.2)	11.3 (0.1)	13.5 (0.2)	10.7 (0.1)	14.4 (0.1)
Monocytes	13.1 (0.1)	16.8 (0.2)	13.4 (0.2)	17.1 (0.2)	12.7 (0.2)	17.7 (0.2)
Lymphocytes	8.4 (0.1)	11.2 (0.1)	8.4 (0.1)	11.6 (0.1)	8.2 (0.1)	11.3 (0.1)
Ovoid Thrombocytes	9.5x5.7 (0.1, 0.01)	12.7x6.3 (0.2, 0.1)	9.6x5.7 (0.1, 0.1)	13.1x6.7 (0.2, 0.1)	9.4x5.5 (0.2, 0.1)	3.1x6.5 (0.2, 0.1)
Spindle Thrombocytes	12.8x3.8 (0.1, 0.1)	16.2x4.4 (0.1, 0.1)	12.9x3.7 (0.1, 0.03)	16.3x4.5 (0.1, 0.1)	12.7x3.8 (0.1, 0.1)	16.3x4.4 (0.1, 0.1)
Spiked Thrombocytes	18.0x4.9 (0.1,0.1)	23.1x5.1 (0.3, 0.2)	18.8x4.5 (0.6, 0.1)	22.9x5.2 (0.3, 0.1)	18.1x4.8 (0.4, 0.2)	22.2x5.7 (0.4, 0.2)

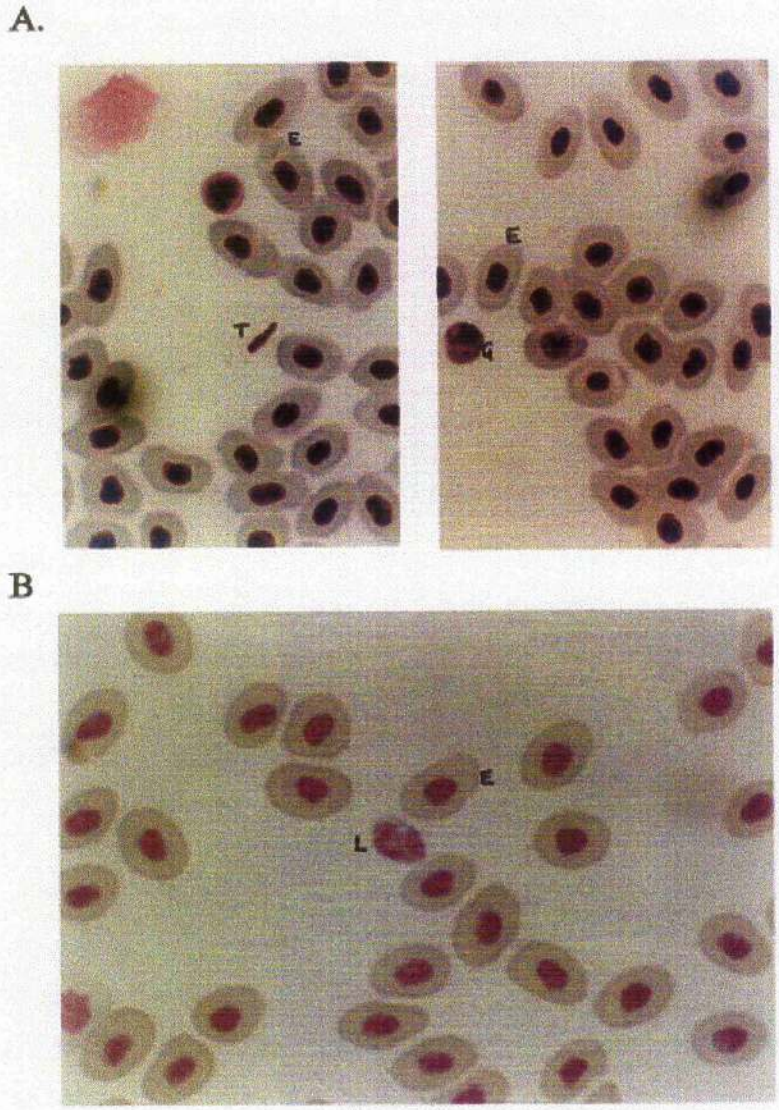


Figure 2.2 Peripheral blood granulocytes (G), lymphocytes (L), thrombocytes (T) and erythrocytes (E) of diploid (A i, ii) and triploid (B) Atlantic salmon.

Table 2.5 Diploid/triploid ratio of peripheral blood leucocyte sizes for 3 families of sibling diploid and triploid Atlantic salmon

	Family J20 mixed sex	**Family O18 all-female**	**Family I18 mixed sex**
Granulocytes	0.82	0.84	0.74
Monocytes	0.78	0.78	0.71
Lymphocytes	0.75	0.72	0.72
Ovoid Thrombocytes	0.75 x 0.9	0.73 x 0.85	0.72 x 0.85
Spindle Thrombocytes	0.79 x 0.86	0.79 x 0.82	0.78 x 0.86
Spiked Thrombocytes	0.78 x 0.96	0.82 x 0.86	0.82 x 0.84

2.4 Discussion

2.4.1 Packed cell volume

In this study the percentage of total blood volume occupied by cells was measured and has been referred to as 'packed cell volume'. As can been seen from the cell counts, the packed cell volume is mostly made up of erythrocytes with leucocytes occupying 1% or less of the total blood volume. In many studies within the literature, the term 'haematocrit' has been used to refer to 'packed cell volume' (eg. Benfey, 1997), although by strict definition 'haematocrit' is the percentage of total blood volume occupied by erythrocytes.

The literature states that diploid and triploid fish do not have significantly different haematocrits (Aliah *et al.*, 1991; Benfey, 1997; Benfey and Sutterlin, 1984; Biron and Benfey, 1994; Graham *et al.*, 1985; Small and Randall, 1989; Yamamoto and Iida, 1994). The only exception to this is Virtanen *et al.* (1990) who found that triploid rainbow trout had a lower haematocrit than comparative diploids.

In this study, on 2 out of the 3 sampling occasions there was no difference in packed cell volume (PCV) between diploid and triploid Atlantic salmon. The PCV's for these two occasions and for triploids in February 1996 ranged from 43-49% which are within the normal range for Atlantic salmon (Elliot *et al.*, 1966). However, the mean PCV for the diploids sampled in February 1996 was 57% which is higher than the normal range stated in the literature. This is a similar result to that found by Virtanen *et al.*, (1990). Increased PCV can occur for many reasons; for example, disease, stress, nutrition (Biron and Benfey, 1994; Blaxhall, 1972; Blaxhall and Daisley, 1973; Elliot, *et al.*, 1966), although Biron and Benfey (1994) did not find that stress caused the PCV of diploid and triploid brook trout to be different. However, PCV can also vary considerably between sampling, with season and with environmental conditions (Blaxhall, 1972; Toney and Coble, 1980).

The cause of increased packed cell volume, in the February 1996 sample for diploids, is unknown and it is also unknown why there was not a corresponding increase in triploid packed cell volumes. However, when considering all the data generated in this study it can be concluded that the total blood volume occupied by cells is not different between diploids and triploids.

The range of PCV within a healthy population can be large (Blaxhall, 1972). Figure 2.1 shows the range of PCV in all sample groups. The range does not differ between diploids and triploids except in the February 1996 sample in which the diploid groups have a larger range.

2.4.2 Cell counts

Total blood cell and erythrocyte numbers were reduced in the triploid salmon which concurs with the literature on other fish species (Aliah, *et al.*, 1991; Benfey, 1997; Benfey and Sutterlin, 1984; Graham, *et al.*, 1985; Yamamoto and Iida, 1994). However, many authors state that cell numbers are one third lower in triploid fish in comparison to diploids (reviewed in (Benfey, 1997)). This was not found to be the case in this study. Triploid cells were even fewer in number with a ratio of 1.88:1, diploid to triploid cells. Therefore there are almost 50% fewer erythrocytes in the triploids studied. This suggests that triploid cells are more than one third larger than diploid cells.

Blood leucocyte numbers were also reduced in the triploid fish. Although Small and Benfey (1987) reported that polymorphonuclear leucocytes in triploid Atlantic salmon were increased in size, they did not report on leucocyte numbers in triploids. Yamamoto and Iida (1994) showed that triploid rainbow trout neutrophils were less numerous than diploid ones in whole blood. Similar to total cell and erythrocyte numbers, this study shows that triploid leucocytes are more than one third fewer in number than diploid leucocytes, suggesting that triploid leucocytes are more than one third larger than diploid leucocytes. The ratio of diploid to triploid leucocyte numbers was 1.69:1 which equates to 41% fewer leucocytes in triploids rather than the theoretical 33%.

2.4.3 Cell sizes

It is well documented that erythrocytes are larger in triploids (Benfey, 1997; Benfey and Sutterlin, 1984; Graham, *et al.*, 1985; Small and Benfey, 1987). Various other cell types, including brain, retinal, epithelial, muscle, liver and kidney cells, have also been shown to be larger in triploid fish (Benfey, 1997; Small and Benfey, 1987). Many authors state that triploid cells are one third larger.

Small and Benfey (1987) studied the size of polymorphonuclear leucocytes in Atlantic salmon and found them to be larger in triploids. It is not evident whether these leucocytes were from peripheral blood or in tissues. There are no studies within the literature which report on the size of other leucocytes from triploid fish.

In this study, granulocytes (neutrophils), lymphocytes, thrombocytes and monocytes within peripheral blood, were all larger in triploids. Theoretically, if triploid cells were one third bigger, the triploid: diploid ratio would be 1:0.66. However, the average ratios are 1:0.8, 0.76, 0.73, 0.73 and 0.79 for granulocytes, lymphocytes, monocytes, ovoid thrombocytes, spindle thrombocytes and spiked thrombocytes respectively.

Therefore, instead of being 33% larger than diploid cells, the triploid leucocytes in this study are approximately 20% larger than diploids. This is slightly smaller than theoretically expected.

Although measurements of cell size from blood smears is not ideal (because the physical smearing of cells and changes in shape and size upon adherence to the slide, and also that this only gives a 2 dimensional view of the cell) it is clear that triploid cells are larger. However, it may not reflect the true dimensional changes in living cells. Also the fixation method used on this occasion may alter cell shape and size. Fixation causes cell shrinkage and considering the differences in cell size between diploids and triploids, it is possible that triploid cells shrink more and more quickly than diploid cells. This may account for the factor of difference in cell sizes not corresponding with that presented in the literature.

It is difficult to estimate the difference in size between diploid and triploid cells from blood smears. This is because of the irregular shape of cells and the variety of shapes which can occur within a particular cell type. There is also no estimation of cell height. From studies on erythrocytes and other cells, it appears that increased cell size is not uniform in all dimensions but that there is a greater increase in

cellular length than in width (Benfey, 1997; Benfey and Sutterlin, 1984; Graham, *et al.*, 1985; Small and Benfey, 1987). Some of these studies have concluded that cell height does not change between diploid and triploid cells but Benfey (1997) suggests that this should be viewed with caution as cell height is derived rather than measured.

If increased cell length and width is not accompanied by increases in cell height, triploid cells should have an even lower surface area: volume ratio and any function limited by surface area should be consequently reduced in triploids. However, this conclusion does not account for potential changes in the cell shape. It is interesting to note that studies of polyploid cell populations have shown that those cells which possess nuclei with highest ploidy also have highly indented cell membranes (Brodsky and Uryvaeva, 1978), and therefore the assumption of decreased surface area in triploid cells may be inaccurate. It was not possible to determine whether the cell membrane of triploid leucocytes was more indented than that of diploid leucocytes in this study.

These investigations into cell sizes have suggested that triploid leucocytes are smaller than theoretically expected. However, as discussed above there are a number of problems associated with measuring cell size. In contrast, haematocrits and cell counts are not differentially affected by sample processing. Derivation of cell size from cell counts and packed cell volume is therefore probably more accurate. These parameters were not measured for the same groups of fish in this study, so derivation would be inappropriate in this case. However, considering that haematocrits are comparable between diploids and triploids, the results of the study into cell numbers of diploid and triploid Atlantic salmon found that triploid erythrocytes were 50% fewer in number, rather than the expected 33%. This suggests that they are larger than expected (ie. more than 33% larger than diploid erythrocytes). Leucocytes were 41% fewer in triploids, which although is not far from the theoretical 33%, also suggests that these cells are slightly larger than expected (ie. more than 33% larger than diploid leucocytes).

3. A comparison of aspects of the non-specific, humoral immune system between diploid and triploid Atlantic salmon

3.1 Introduction

Non-specific, humoral defence mechanisms include microbial growth inhibitory substances, enzyme inhibitors, lysins, agglutinins and precipitins (Alexander and Ingram, 1992). In this study a selection of these were examined.

3.1.1 Iron with-holding mechanisms

Microbial growth inhibitory substances include acute phase proteins and interferons. Proteins such as transferrin, caeruloplasmin and metallothionein, chelate metal ions and therefore deprive bacteria and other parasites of essential inorganic ions (Alexander and Ingram, 1992; Roed *et al.*, 1995). Transferrin is an iron-binding protein and is one element of the mechanism termed iron with-holding (Weinberg, 1984; Weinberg, 1992). This system attempts to deny invading organisms and neoplastic cells of iron whilst permitting normal host cells access.

Iron with-holding is one of a range of non-specific defence mechanisms present in most vertebrates. Iron with-holding mechanisms are both cellular and non-cellular, and these strategies can be classified into two groups; constitutive measures and induced components.

Constitutive measures guard against potential invasion of bacteria by the strategic placement of iron-binding proteins. Conalbumin (also known as ovotransferrin) is present in eggs in a highly unsaturated state (Weinberg, 1984). Lactoferrin is present in exocrine secretions of mammals (eg. milk, tears, saliva, nasal exudate, bile etc) so is present at many sites of potential invasion by pathogens (Otto *et al.*, 1992; Weinberg, 1984). Blood neutrophils of mammals also contain high levels of lactoferrin (Bullen and Armstrong, 1979).

Induced components of the system are activated by cytokines such as interleukins 1 and 6, and tumour necrosis factor (TNF) (Weinberg, 1992). These induced responses include

- suppression of intestinal assimilation of iron (Weinberg, 1992)

- retention of iron by macrophages which have digested haemoglobin from decaying erythrocytes and which prevents its normal recycling to transferrin. Macrophages continue to acquire iron from decaying erythrocytes and quickly initiate synthesis of ferritin, an iron storage protein, to allow for intracellular storage of additional iron (Ramsay, 1958; Weinberg, 1989; Weinberg, 1992).

- Reduction in expression of transferrin receptors on activated macrophages which reduces the amount of iron in the cytoplasmic pool of these cells, hence reducing availability to phagocytosed pathogens (Brock, 1995).

- mobilisation of neutrophils to sites of invasion. Lactoferrin is a major component of the specific granules found in polymorphonuclear neutrophilic leucocytes (Bullen and Armstrong, 1979; Otto, *et al.*, 1992; Weinberg, 1984). It is released into septic areas upon degranulation of these leucocytes, and after binding with iron in the infected region the saturated protein is ingested by macrophages which then remove or store the iron on ferritin (Weinberg, 1984; Weinberg, 1992).

- lowering of plasma or serum iron levels and diversion of plasma iron to liver (Weinberg, 1984; Weinberg, 1992).

- Lactoferrin has been reported to affect various other immune functions including production of cytokines, natural killer cell cytotoxicity, complement activation and lymphocyte proliferation (Brock, 1995) and may also aid bactericidal killing by assisting iron to catalyse the formation of

hydroxyl radicals (Weinberg, 1984).

Transferrin is found in various body fluids but is most abundant in plasma and lymph (Otto, *et al.*, 1992; Weinberg, 1984). Transferrin, unlike conalbumin and lactoferrin, does not function solely as an iron with-holding protein; it is also involved with iron transport. Therefore, *in vivo*, whereas conalbumin and lactoferrin are highly unsaturated, transferrin has a higher percentage saturation. Saturation in mammals varies with age and species (Weinberg, 1984) and differences in saturation can cause differences in bacteriostatic effect. For example, normal human serum was found to inhibit growth of *Mycobacterium avium* in human macrophages. However in the presence of holotransferrin (iron-saturated transferrin) there was no inhibition but apotransferrin (iron-free transferrin) inhibited growth (Douvas *et al.*, 1993). Lactoferrin is usually highly unsaturated (Weinberg, 1992) and thus has a high iron-binding capacity.

Unlike lactoferrin and conalbumin, transferrin does not function at low pH (Weinberg, 1984). This restricts its usefulness in septic areas where pH has been lowered by the release of lactic acid by host and pathogenic cells. Lactoferrin is able to bind iron under acidic conditions due to the possession of a strongly basic region close to the binding area (Brock, 1995; Weinberg, 1984). When lactoferrin binds with iron it becomes more resistant to proteolytic degradation (Brock, 1995) which also allows it to function in septic areas where proteinases are released from host and pathogenic cells. Congleton and Wagner (1991) have shown that fish exhibit an acute phase hypoferraemic response. The mechanism by which the hypoferraemic response is initiated in fish is unknown but in mammals it is mediated by interleukin-1 (IL-1). During an acute phase response elevated IL-1 levels occur and stimulate the release of lactoferrin by neutrophils (Klempner *et al.*, 1978). The lactoferrin removes iron from serum transferrin forming lactoferrin-iron complexes which are rapidly cleared from circulation by the liver where the iron is stored as ferritin (Weinberg, 1992).

Transferrin, lactoferrin and conalbumin are homologous proteins which together are often referred to as the transferrins. A homology also exists between the transferrins and caeruloplasmin (Bezkorovainy, 1987). Human transferrins can bind two atoms of ferric iron per molecule of protein (Bezkorovainy, 1987). The binding of iron is accompanied by the binding of an anion (usually a carbonate). Iron is not bound in the absence of an anion (Bezkorovainy, 1987). A transferrin molecule has two domains which are termed the 'N-terminal' and 'C-terminal' domains. Each domain has differing binding properties; the absorption spectra are different with hen egg conalbumin N and C domains having maximum absorbance at 450nm and 430nm respectively and the whole molecule 440nm; responses to pH differ with the N-terminal being acid labile and the C-terminal being acid stable (Bezkorovainy, 1987). However, despite some debate, the two binding sites are considered to be functionally equivalent. There are 4 forms of transferrin; apo-transferrin, transferrin carrying iron on either the N or C teminus and diferric transferrin; each occurs in different frequencies (Bezkorovainy, 1987).

Bacteria have evolved several mechanisms to overcome the ability of the host to withhold iron. The best studied system is that of iron chelators, called siderophores which compete with lactoferrin and transferrin for iron. Bacterial iron regulated outer membrane proteins (IROMPS) usually serve as receptors for iron-siderophore complexes or directly for host transferrin and are essential for some mechanisms of iron uptake (Otto, *et al.*, 1992).

Typical strains of *Aeromonas salmonicida* grown in iron restricted conditions were able to sequester iron from iron-binding proteins by two mechanisms: siderophore and siderophore-independent systems (Chart and Trust, 1983). Atypical strains did not appear to produce a siderophore. Hirst *et al.* (1991) also found that both typical and atypical strains of *A. salmonicida* grow and multiply under conditions of iron restriction imposed by transferrin, however they used different iron uptake mechanisms (siderophore mediated and siderophore independent respectively). The siderophore independent mechanism was suggested by Chart and Trust (1983) to involve direct contact between the cell surface and an iron-binding protein without

the production of a siderophore. However, Hirst and Ellis (1996) have shown that the non-siderophore mediated mechanism for sequestering iron from transferrin is mediated by an extracellular proteinase.

Photobacterium damsela uses a siderophore-mediated mechanism to obtain iron and can grow in iron limited conditions (Magarinos *et al.*, 1994). Some strains of *Vibrio anguillarum* possess a plasmid which is associated to virulence, and possession of this plasmid appears to allow the pathogen to grow in iron limited conditions. In contrast, the growth of plasmidless strains appears to be inhibited by the presence of iron-binding proteins (Crosa, 1984). Radioactive iron uptake experiments show that this is due to a more rapid and efficient iron uptake mediated by the virulence plasmid. In addition, *V. anguillarum* cells growing under iron limited conditions show at least two extra outer membrane proteins (OMPs) (Crosa, 1984).

3.1.2 Enzyme inhibitors

Enzyme inhibitors inhibit the function of otherwise potentially hazardous enzymes that may be secreted by an invading organism. Those so far detected in fish appear to be principally anti-microbial. These are mainly antiproteases such as α_1-proteinase inhibitor (previously known as α_1-antitrypsin) and α_2 macroglobulin (Alexander and Ingram, 1992; Ellis *et al.*, 1981).

Many pathogens have been shown to produce toxins *in vitro* in the form of extracellular products (ECP's) which include extracellular proteinases (EP's). Evidence is also accumulating which suggests that the growth and proliferation of pathogenic organisms depends on these proteinases (Travis *et al.*, 1995). Studies have been carried out on the ECP's of *Aeromonas salmonicida* (Ellis *et al.*, 1997; Ellis, *et al.*, 1981; Fyfe *et al.*, 1988; Fyfe *et al.*, 1986; Kawahara *et al.*, 1990), *Vibrio* spp. (Nottaga and Birbeck, 1987) and *Henneguya salmonicida* a myxosporean parasite of salmonids (Bilinski *et al.*, 1984). With *Vibrio* and *Aeromonas* the ECP's are toxic and are suggested to lead to the death of the fish, whereas with the myxosporidian they digest fish muscle to allow the parasite to feed and spread. Serum from healthy rainbow trout reduced the toxicity of ECPs (Ellis, *et al.*, 1981).

The injection of inhibitors has been found to increase the fish's natural protection against bacterial exotoxins (Salte *et al.*, 1993).

Serum contains many serine proteinase inhibitors, including α1-proteinase inhibitor (also known as α_1-antitrypsin), antithrombin III, α_2-antiplasmin 1, α-antichymotrypsin, C1-inhibitor and inter α-trypsin inhibitor. Most serine proteinase inhibitors contain a target region which mimics the enzyme's substrate. In doing so it becomes irreversibly bound to the enzyme which is inactivated and the complex digested by phagocytes (Alexander and Ingram, 1992).

Alpha-1-proteinase inhibitor is known in mammals to inactivate almost all serine proteinases. It is composed of a single polypeptide chain with two disulphide bonds (Alexander and Ingram, 1992). Alpha-2-macroglobulin binds to all classes of proteinase (Alexander and Ingram, 1992; Barrett and Starkey, 1973; Travis and Salvesen, 1983). The α_2 macroglobulins are large molecular weight proteinase inhibitors which "cage" proteinases, rather than bind them, limiting their ability to hydrolyze protein substrates. However, it does not inhibit proteinase activity against low molecular weight amide or ester substrates since it does not affect the active site of the proteinase, and being of low molecular weight the substrate can enter the 'cage'for access to the proteinase (Alexander and Ingram, 1992; Barrett and Starkey, 1973; Travis and Salvesen, 1983).

Alpha-2-macroglobulin (α_2M) may play a defensive role in some diseases. α_2M from the serum of several species of salmonids has been shown to be an effective inhibitor of EPs produced by *Aeromonas salmonicida* (Ellis, 1987; Freedman, 1991; Salte, *et al.*, 1993). In mammals, fibroblasts and macrophages possess α_2M receptors, which recognise and then internalise proteinase: α_2M complexes (Travis, *et al.*, 1995). Antithrombin (AT) has also been shown to have an inhibitory role against *A. salmonicida* EPs (Salte, *et al.*, 1993). AT was directed against generated thrombin and activated factor X, whereas α_2M inhibited the proteinase directly.

3.1.3 Lysozyme

Lysins are substances which cause cell lysis. These fall into several categories; hydrolases, proteinases and non-specific lysins (Alexander and Ingram, 1992). In fish, the most common hydrolase is N-acetylmuramide glycanohydralase better known as lysozyme or muramidase, but chitinase and chitobiase also occur (Alexander and Ingram, 1992). Unlike mammals, fish seem to posses lysozyme that has anti-microbial activity against Gram-negative and Gram-positive bacteria (Yano, 1996). Lysozyme is distributed mainly in tissues rich in leucocytes, in areas of potential bacterial invasion and in eggs (Grinde, 1989; Ourth, 1980; Yousif *et al.*, 1994). Lysozyme has been shown in neutrophils and monocytes (Murray and Fletcher, 1976) and it has also been speculated that they may contribute to serum lysozyme levels (Yano, 1996). This evidence suggests that lysozyme is a constitutive defensive strategy employed by the fish, as well as a defensive measure that can be used in response to infection or injury.

3.1.4 Complement

The complement system is described in Chapter 1.3.3. Teleost fish have the alternative and classical complement pathways (ACP and CCP respectively) comparable to the mammalian complement system. In the CCP, C1 is activated by an antigen-antibody complex and results in a cascade of complement components being activated; C4, C2, C3, C5, C6, C7, C8 and C9. The ACP is initiated by the direct activation of C3 and the cascade follows as with the CCP. C3 has to be in the presence of factors B and D for the activation of ACP. Complement activity (initiated by either the ACP or the CCP) results in

- Smooth muscle contraction and increased capillary permeability, mediated by C3a and C5a (Yano, 1996)
- Enhanced phagocytosis and bacterial killing by opsonisation, mediated by C3 (Sakai, 1992)
- Chemotaxis of leucocytes to areas of infection or injury by complement components (eg. C5a) (Sakai, 1992; Secombes, 1996; Yano, 1996).

- Cell lysis by the formation of the membrane attack complex (MAC) from C5, C6, C7, C8 and C9 with C5b forming the nucleus (Sakai, 1992; Secombes, 1996; Yano, 1996).

Complement has been shown to have anti-viral, anti-microbial and parasiticidal activity in fish (Yano, 1996). Many of the complement components and products identified in mammals have also been identified in teleost fish including C3, C5 and MAC in rainbow trout (Yano, 1996). The activity of serum complement maintains a defined constant level in healthy individuals, but can fluctuate in response to physiological and pathological changes. These changes include seasonal variation (Yano, 1996) and sexual maturation (Roeed *et al.*, 1992). Complement activity has also been detected in skin mucus of rainbow trout (Yano, 1996).

3.1.5 Objectives

It was the purpose of these experiments to determine baseline levels of various immune parameters in healthy diploid and triploid individuals.

3.2 Materials and Methods

3.2.1 Fish

Experiment A.

Initial investigations were made into serum lysozyme, α_2 macroglobulin, total anti-trypsin, classical complement activity. Each parameter was investigated separately using one out-bred stock of sibling, all-female diploid and triploid Atlantic salmon (average weight approx. 1.2kg) from the FRS Fish Cultivation Unit, Aultbea. The diploids and triploids were kept in separate tanks (1m diameter) supplied with flow through sea water at ambient temperature (6.9°C at the time of sampling). They were fed 2% body weight daily with Trouw Mainstream non-pigmented salmon diet

Experiment B.

One out-bred stock of sibling, all-female diploid and triploid Atlantic salmon from the FRS Fish Cultivation Unit, Aultbea were used (average weight approx. 800g). This was a different year class to the stock used in Experiment A. The diploids and triploids were kept in separate tanks (1m diameter) supplied with flow through sea water at ambient temperature (6.1°C at time of sampling). They were fed 2% body weight daily with Trouw Mainstream non-pigmented salmon diet. Twenty individuals from each group were sampled (20 2n and 20 3n fish) for serum. The samples were used to determine α_2 macroglobulin and total anti-trypsin activity, total iron content and iron-binding capacity, and alternative pathway complement activity. A comparison of these parameters between diploid and triploid fish was made in order to confirm results from experiment A, and since, on this occasion, all these parameters were measured in most of the individuals from the same sample, an investigation was also made to determine whether there was any relationship between the parameters.

3.2.2 Serum Collection

The fish were anaesthetised by immersion in tricaine methanesulfonate (MS-222, Sigma) at a final concentration of 2mg l^{-1} and blood collected from the caudal vein using a serum sep vacuette (Greiner) and 23G needle. Blood was allowed to clot for at least one hour at ambient temperature and centrifuged at 1431g for 20 min. Serum was then aliquoted, transported on dry ice and stored at -80°C until required.

3.2.3 Non-specific, Humoral Assays

Classical Pathway Complement Activity.

This assay was adapted from Yano (1992) and miniaturised for use in microtitre plates to make the assay more efficient of time and sample.

The following buffers were used: veronal buffered saline-VBS (Sigma Barbital Buffer B6632 adjusted to 0.006M pH7.2, with 150mM sodium chloride); metal stock solution (7.35% calcium chloride dihydrate (w/v) and 20.33% magnesium chloride hexahydrate (w/v) in distilled water); gelatin veronal buffer-GVB (VBS

with 0.1% gelatin (w/v), and 0.1% (v/v) metal stock solution) and EDTA-GVB (0.1% gelatin (w/v) and 0.01M ethylenediaminetetraacetic acid in VBS).

An agglutination assay was performed on each new batch of salmon anti-SRBC antisera (supplied by Dr. Houghton, FRS Marine Laboratory, Aberdeen) to determine the optimal dilution of antisera required to sensitise the sheep red blood cells (SRBC's). SRBC's were washed in VBS by centrifuging at 1100g for 5 min, three times. The SRBC's were then diluted to a 3% (v/v) suspension in GVB. The antiserum was heat-inactivated (45°C for 20 min) and serially diluted in 50µl GVB and 50µl of 3% SRBC's was added. After 2h the sub-agglutinating titre was determined and this dilution was used as the optimal dilution for the antiserum in the preparation of the erythrocyte-antibody (EA) suspension.

SRBC's were washed in VBS and suspended to a 3% concentration in heat-inactivated anti-serum optimally diluted in EDTA-GVB. The anti-sera-SRBC suspension was allowed to stand for 1 h at 22°C to sensitise the cells. The SRBC's were then washed in GVB and resuspended to the same volume.

Fifty µl of test serum was serially diluted in GVB in a U-well microtitre plate in replicates of four. Two replicates were exposed to 50µl of the 3% EA suspension and two were processed as sample blanks with GVB in place of EA suspension. Positive controls of 50µl distilled water and 50µl 3% EA suspension (for 100% haemolysis), negative controls of 50µl GVB and 50µl 3% EA suspension (for spontaneous haemolysis), and blanks of 100µl GVB were also made. The plate was incubated for two hours at room temperature then centrifuged to remove any remaining cells from the supernatant, which was then transferred to a clean microtitre plate and the optical density read at A450nm on a microplate reader. The percentage lysis for each sample and dilution was calculated using the 100% lysis positive control. The reciprocal dilution giving 50% lysis (CH_{50}) could then be determined by PROBIT analysis using GENSTAT.

Alternative Pathway Complement Activity.

This assay was adapted from Yano (1992) and miniaturised for use in microtitre plates to make the assay more efficient of time and sample.

The following buffers were used: veronal buffered saline-VBS (Sigma Barbital Buffer B6632 adjusted to 0.006M pH7.2, with 150mM sodium chloride); gelatin veronal buffer-GVB (VBS with 0.1% gelatin (w/v)), EGTA-Mg-GVB and EDTA-GVB (0.1% gelatin (w/v) and 0.01M ethylenediaminetetraacetic acid in GVB).

A suspension of rabbit blood diluted 50% in Alsevers (SAPU) was used, and kept at 4°C. As required, aliquots of blood cells were washed three times with GVB. The cell suspensions were then diluted in EGTA-Mg-GVB and the cell concentration was adjusted to 2 x 10^8 cells/ml. Cell concentrations were counted using a haemocytometer.

Twenty five µl test serum was serially diluted in GVB in a U-well microtitre plate in replicates of four. Two replicates were exposed to 25µl of the RaRBC suspension and two were processed as sample blanks with EGTA-Mg-GVB in place of RaRBC suspension. Positive controls of 25µl distilled water and 25 µl RaRBC suspension (100% haemolysis), negative controls of 25 µl EGTA-Mg-GVB and 25µl RaRBC suspension (spontaneous haemolysis), and blanks of 100µl EGTA-Mg-GVB were also made. The plate was incubated for 2h at 22°C then the reaction was stopped by the addition of 140µl EDTA-GVB to every well. The plates were centrifuged, the supernatant transferred to a clean microtitre plate and the optical density read at A415nm on a microplate reader. The percentage lysis for each sample and dilution was calculated using the 100% lysis positive control and plotted. The dilution giving 50% lysis (ACH_{50}) could then be determined by PROBIT analysis using GENSTAT.

Assays for Antiproteinase Activity

The following reagents were used to measure $\alpha_2$macroglobulin and total anti-trypsin activity: 0.1M Tris-HCl buffer pH8; 1mg trypsin /ml HCl stock solution (Boehringer Mannheim) diluted 1:30 with distilled water immediately prior to use; 1mg soya

bean trypsin inhibitor (SBTI, Boehringer Mannheim) /ml distilled water stock solution diluted 1:5 immediately prior to use; 1.9mM Chromozym$^{@TRY}$ solution (Boehringer Mannheim); Atlantic salmon serum (diluted 1:20).

Measurement of $\alpha_2$macroglobulin activity

This method is as described by Boehringer Mannheim. Fifty µl of test serum was diluted in 2ml of Tris-HCl buffer in disposable 1.5ml cuvettes. A control was also carried out by replacing the test sample with distilled water. Fifty µl of trypsin solution was then added to both the test and control. This was mixed and incubated for 1 min at room temperature. Fifty µl of SBTI solution was added to the test and 50µl distilled water was added to the control, mixed and then incubated at room temperature for a further 30 seconds. Two hundred µl of Chromozym$^{@TRY}$ solution was added to both test and control, mixed and the absorbance read every minute for 10 min at A405nm using an Ultraspec III spectrophotometer and enzyme kinetics software (Pharmacia).

The rate of change in absorbance per minute (ΔA/min) was calculated from the linear part of the curve and the activity of the test sample calculated as follows:

Activity Units/ml serum

= (total vol/ (extinction coeff x sample vol. x light path) x ΔA/min) x dilution factor

= (4.159230769 x ΔA/min) x 20

Measurement of total anti-trypsin activity

Fifty µl of test serum was diluted in 2ml of Tris-HCL pH8 buffer in disposable 1.5ml cuvettes. A control was also carried out by replacing the test sample with distilled water. Fifty µl of trypsin solution was then added to both the test and control. This was then mixed and incubated for 1 min at room temperature. Fifty µl of distilled water was added to the test and control, mixed and then incubated at room temperature for a further 30 seconds. Two hundred µl of Chromozym$^{@TRY}$ solution was added to both test and control, mixed and the absorbance read every minute for 10 min at A_{405nm} using an Ultraspec III spectrophotometer and enzyme kinetics software (Pharmacia).

The rate of change in absorbance per minute (ΔA/min) was calculated from the linear part of the curve and the activity of the test sample calculated as follows:

Activity Units/ml serum

= (total vol/ (extinction coeff x sample vol. x light path) x ΔA/min) x dilution factor

= (4.159230769 x ΔA/min) x 20

Measuring total iron content and unsaturated iron binding capacity

Total iron content and unsaturated iron-binding capacity were determined using commercially available reagents (Sigma No. 565).

Total iron (TI) content assay

Fifty µl of serum, standard (iron standard solution 5 µg/ml) or blank (distilled water) were added to 250µl of buffer (acetate buffer pH4.5, with 1.5% hydroxylamine hydrochloride) in a flat well microtitre plate (Greiner). The microplate was shaken and the absorbance read spectrophotometrically with a Dias microplate reader (Dynatech) at A560nm to give the initial readings. Five µl of iron colour reagent (0.85% w/v ferrozine in hydroxylamine hydrochloride) was then added to every well and the plate incubated at room temperature with continuous, gentle shaking. After 10 min the microplate was read again at A560nm to give the final readings. The total iron content was calculated as follows:

change in absorbance of test ΔA_{test} = final A_{test} - initial A_{test}

change in absorbance of standard ΔA_{std} = final A_{std} - initial A_{std}

TI (mg/ml) = $(\Delta A_{test} / \Delta A_{std}) \times 5$

where 5 is the concentration of the iron standard(mg/ml)

Unsaturated iron-binding capacity (UIBC) assay

Fifty µl of serum was added to 200 µl UIBC buffer (0.5M Tris buffer, pH 8.1 with added surfactant, Sigma) in a flat well microtitre plate. Fifty µl of iron standard was then added to all the test samples. Blanks consisted of 100µl of distilled water with 200µl of UIBC buffer and standards were 200µl of UIBC buffer with 50µl iron standard and 50µl distilled water. The microtitre plate was then shaken and the absorbance read at A560nm to give the initial readings. Five µl of iron colour reagent was then added to every well and the plate incubated at room temperature with continuous, gentle shaking to ensure thorough mixing in all wells. After 10 min the microplate was read again at A560nm to give the final readings. The UIBC was calculated as follows:

change in absorbance of test ΔA_{test} = final A_{test} - initial A_{test}

change in absorbance of standard ΔA_{std} = final A_{std} - initial A_{std}

UIBC (mg/ml) = 500 - $(\Delta A_{test} / \Delta A_{std}) \times 5$

where 5 is the concentration of the iron standard (mg/ml)

Total iron binding capacity (TIBC) (mg/ml) = TI + UIBC

Percentage saturation (%) = (TI/TIBC) X 100

Measuring serum lysozyme activity

Lysozyme was measured using a turbidimetric assay adapted for 96 well microtitre plates from Ellis (1990). The reagents and stock solutions used in this assay were phosphate buffered saline (PBS) pH 6.4 and 0.4mg/ml suspension of *Micrococcus lysodeikticus* (Sigma).

Twenty five µl of serially diluted serum, 25µl hen egg white lysozyme (HEWL, Sigma) standards (0, 5, 10, 15, 20, 25, 50, 100 units/well), 25µl of PBS as a

negative control and 200µl of PBS for the blanks were plated. *Micrococcus lysodeikticus* suspension (175µl) was then added to all wells except the blanks, and the plate read at A540nm every min for five min.

A standard curve was plotted and the units of lysozyme per well of serum was calculated. Final results were expressed as the number of lysozyme units per millilitre of serum.

3.3 Results

Experiment A

Table 3.1 shows means, standard errors and number of observations for the lysozyme, antiproteinase and classical pathway complement activity.

Lysozyme levels were immeasurably low in serum (n=16 diploids, n=16 triploids) of diploids and triploids. However, serum of rainbow trout, *Onchorynchus mykiss*, and halibut, *Hippoglossus hippoglossus* and hen egg white lysozyme (HEWL) showed positive results.

Antiproteinases were found to be present in serum (n=3 diploids, n=2 triploids). Although there did not appear to be any difference in total anti-trypsin activity of diploids and triploids, the latter had significantly lower levels of α_2 macroglobulin ($p=0.004$).

Classical pathway complement activity was not significantly different between diploids and triploids.

Experiment B.

Data for all individuals for length, weight, haematocrit and non-specific, humoral defence parameters are shown in Table 3.2. One sample of serum from a triploid was lost during processing. Other data are missing due to insufficient serum to complete all assays.

The relationship between length and weight in the two groups, diploids and triploids, was investigated and an outlier in the diploid group was found. This was due to a spinal deformity and the data for this fish has been discounted. The other variables were examined for relationships to length and were found to be independent of length.

Total anti-trypsin and alpha-$_2$-macroglobulin activity were measured in 12 individuals for both groups (see Figure 3.1). A significantly lower activity of α_2 macroglobulin ($p=0.04$) was found in triploids with mean values of 2.52 and 1.74 activity units/ml serum, diploids and triploids respectively. Total anti-trypsin activity was also determined to be significantly lower in triploids ($p=0.071$) in comparison to diploid siblings with mean values of 2.05 and 2.92 activity units/ml, respectively.

Total iron content (TI), unsaturated iron-binding capacity (UIBC), total iron binding capacity (TIBC) and percentage saturation (% sat) were not significantly different between diploid and triploid fish (see Figure 3.2).

Alternative pathway complement activity was not significantly different between the groups ($p=0.865$), with means of 140 and 142 ACH50 units/ml for diploids and triploids respectively.

An investigation was also made to determine whether there were any correlations between the different parameters (ie. if one parameter changed did another change also). The following parameters were compared; length, weight, α_2 macroglobulin and alternative pathway complement activity. There was a highly significant positive correlation between length and weight (2n $r=0.929$, 3n $r=0.9551$). There were no other significant relationships between parameters.

Table 3.1 Experiment A: Mean (and standard deviation) alpha-2-macroglobulin activity (α2m), total anti-trypsin activity (TAT), lysozyme activity and classical pathway complement activity (CH50) of diploid and triploid Atlantic salmon (- indicates 'not measured' ; * indicates 'immeasurably low quantities').

		α_2m units/ml	TAT units/ml	Lysozyme units/ml	CH50 units/ml
Diploids	**Mean**	1.58	1.60	*	385.55
	Std Error	0.40	0.32	*	73.31
	No. Obs	3	3	16	16
Triploids	**Mean**	1.12	01.43.11	*	393.17
	Std Error	0.27	0.11	*	23.22
	No. Obs	2	2	16	16
Halibut	**Mean**	-	-	624	-
(Diploid)	**No. Obs**			6	
Rainbow Trout	**Mean**	-	-	487	-
(Diploid)	**No. Obs**			12	

Table 3.2 Experiment B: Alpha-2-macroglobulin activity (α2m), total anti-trypsin activity (TAT), total serum iron content (TI), unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), percentage saturation of transferrin and alternative pathway complement activity (ACH50) showing means, medians and standard deviations (*=data missing due to insufficent serum; **=data discounted due to spinal deformity; ***=sample lost during processing).

DIPLOID

Fish No.	Weight g	Fork Length cm	α_2m units/ml	TAT units/ml	UIBC mg/ml	TI mg/ml	TIBC mg/ml	% saturation	ACH50 units/ml
1	570	38	1.45	2.98	14.26	4.30	18.56	23	85.09
2 **	**	**	**	**	**	**	**	**	**
3	824	40	0.90	2.71	16.34	3.38	19.72	17	35.87
4	816	41	1.85	2.76	14.89	4.84	19.73	25	117.96
5	730	40	1.90	2.71	3.93	1.97	5.90	33	251.40
6	931	43	2.62	3.53	2.73	4.57	7.31	63	414.62
7	644	39	1.90	1.36	2.34	2.95	5.29	56	197.36
8	1039	45	2.35	2.62	2.81	4.85	7.67	63	154.78
9	841	42	2.89	3.16	16.28	4.52	20.81	22	330.27
10	1100	45	3.07	3.89	1.66	3.67	5.33	69	461.80
11	589	38	4.70	3.34	3.79 *	*	*		26.91
12	469	36	2.60	1.72	2.78	4.36	7.14	61	208.77
13	731	41	3.80	4.25	6.52 *	*	*		32.41
14	635	41 *	*		9.98	3.56	13.54	26	57.39
15	742	40 *	*		8.66	5.65	14.31	39	27.05
16	640	40 *	*		17.57	4.79	22.37	21	41.18
17	818	43 *	*		7.67	2.70	10.37	26	38.28
18	799	41 *	*		8.70	3.73	12.48	30	38.94
19	647	39 *	*		9.28	2.67	11.95	22	102.57
20	655	38 *	*		7.34	4.42	11.76	38	43.39
Mean	**749.47**	**40.53**	**2.62**	**2.92**	**8.29**	**3.94**	**12.60**	**37.29**	**140.32**
Median	**731.00**	**40.00**	**2.49**	**2.87**	**7.67**	**4.30**	**11.95**	**30.00**	**85.09**
Std Err.	**36.17**	**0.54**	**0.30**	**0.24**	**1.22**	**0.24**	**1.41**	**4.32**	**31.31**

TRIPLOID

Fish No.	Weight g	Fork Length cm	α_2m units/ml	TAT units/ml	UIBC mg/ml	TI mg/ml	TIBC mg/ml	% saturation	ACH50 units/ml
1	1062	45	1.67	2.12	7.11	4.31	11.43	38	100.20
2	458	36	0.18	0.72 *	*	*	*		104.36
3	905	42	2.26	5.83	13.93	3.57	17.50	20	69.22
4	894	42	1.22	3.07	12.73	3.82	16.55	23	66.50
5	760	40	1.18	1.09	7.82	4.46	12.29	36	205.80
6	721	40	1.90	1.99	6.27	4.58	10.85	42	217.54
7	743	42	2.03	2.17	4.33	4.23	8.56	49	297.02
8	532	37	1.72	0.99 *	*	*	*		145.17
9	1108	44	2.71	2.26	6.07	4.28	10.35	41	113.72
10	857	42	2.40	1.45	7.14	4.59	11.73	39	170.32
11	859	42	1.99	1.63	6.28	4.51	10.79	42	404.38
12	765	41	1.63	1.27	3.87	3.32	7.19	46	515.06
13	881	43 *	*		7.04	4.19	11.24	37	21.70
14	839	42 *	*		6.90	2.32	9.22	25	16.99
15	680	40 *	*	*	*	*	*	*	
16	975	44 *	*		5.84	4.56	10.40	44	28.41
17	886	42 *	*		7.21	3.52	10.73	33	34.75
18	746	41 *	*		8.61 *	*	*		30.95
19	895	43 *	*		9.68	3.84	13.52	28	40.27
20	854	42 ***	***	***	***	***	***	***	***
Mean	**821.00**	**41.50**	**1.74**	**2.05**	**7.55**	**4.01**	**11.49**	**36.20**	**142.91**
Median	**855.50**	**42.00**	**1.81**	**1.91**	**7.08**	**4.23**	**10.85**	**38.00**	**102.26**
Std Err.	**34.71**	**0.48**	**0.19**	**0.39**	**0.67**	**0.16**	**0.70**	**2.25**	**33.15**

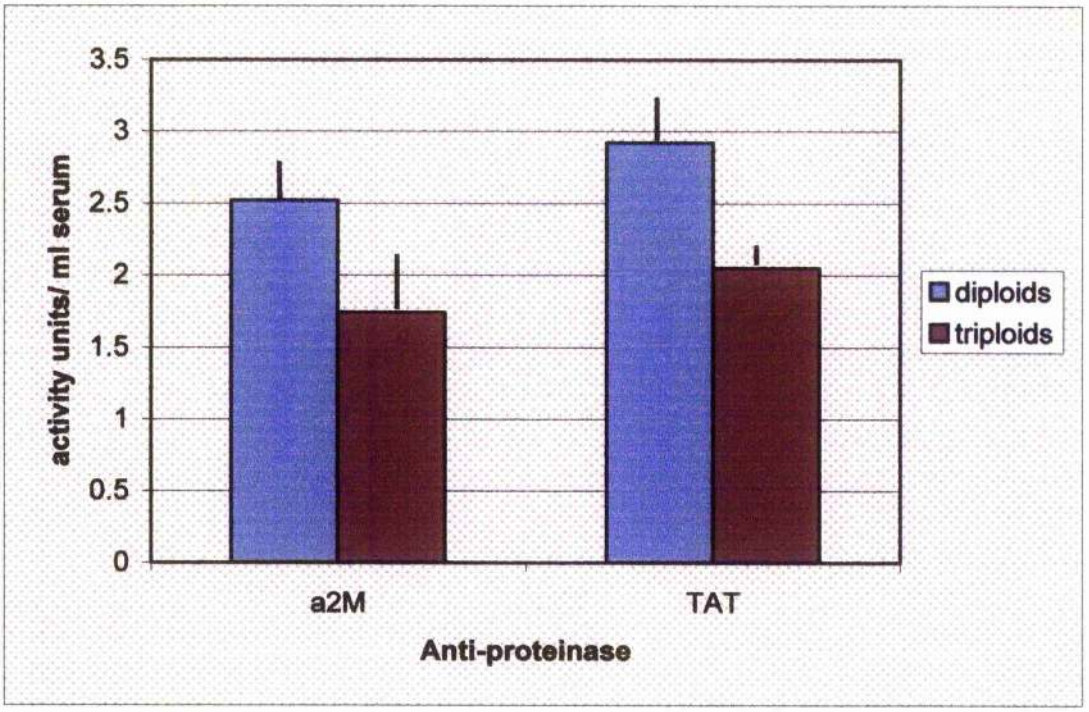


Figure 3.1 Experiment B: Mean antiproteinase activity of diploid and triploid all-female, Atlantic salmon (alpha-2-macroglobulin (α2m) and total anti-trypsin (TAT) activity) showing standard error.

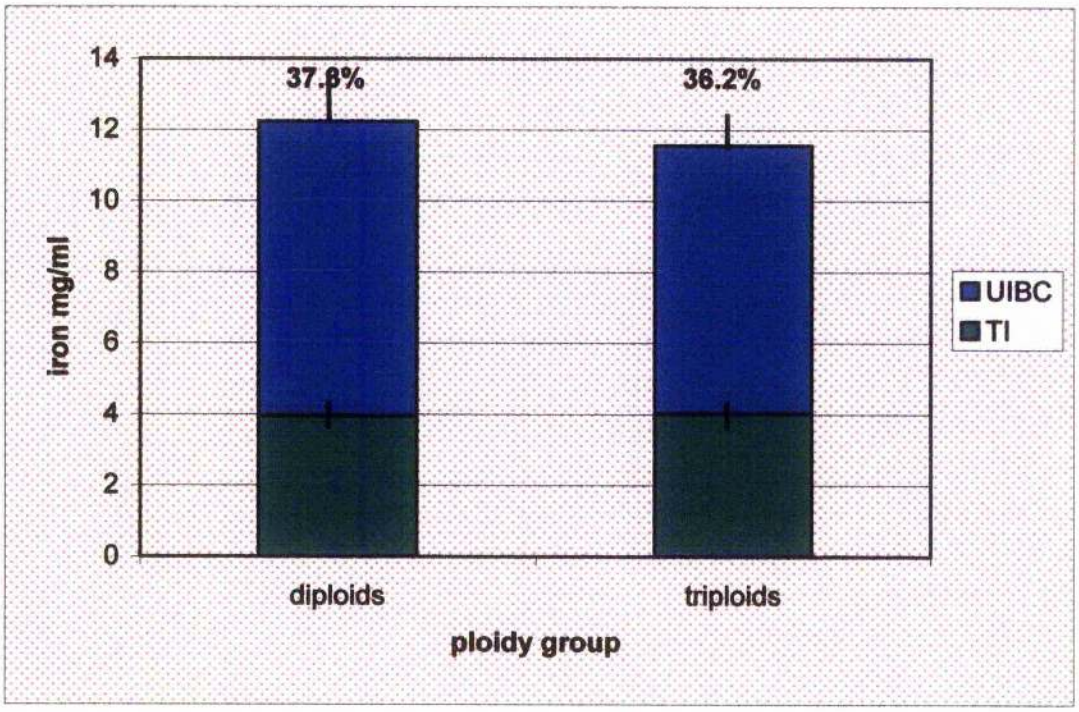


Figure 3.2 Experiment B: Mean total iron content (green), unsaturated iron binding capacity (blue), total iron binding capacity (blue+green) and percentage saturation of diploid and triploid, all-female Atlantic salmon, showing standard error.

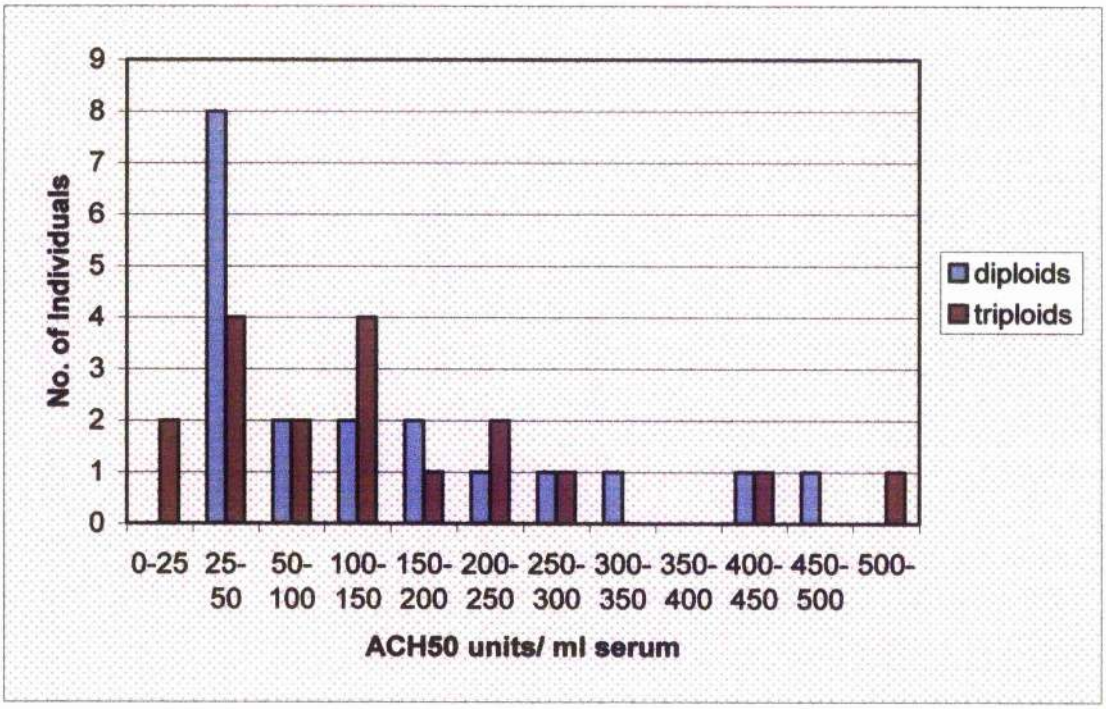


Figure 3.3 Experiment B: Frequency distribution of alternative pathway complement activity in diploid and triploid, all-female Atlantic salmon.

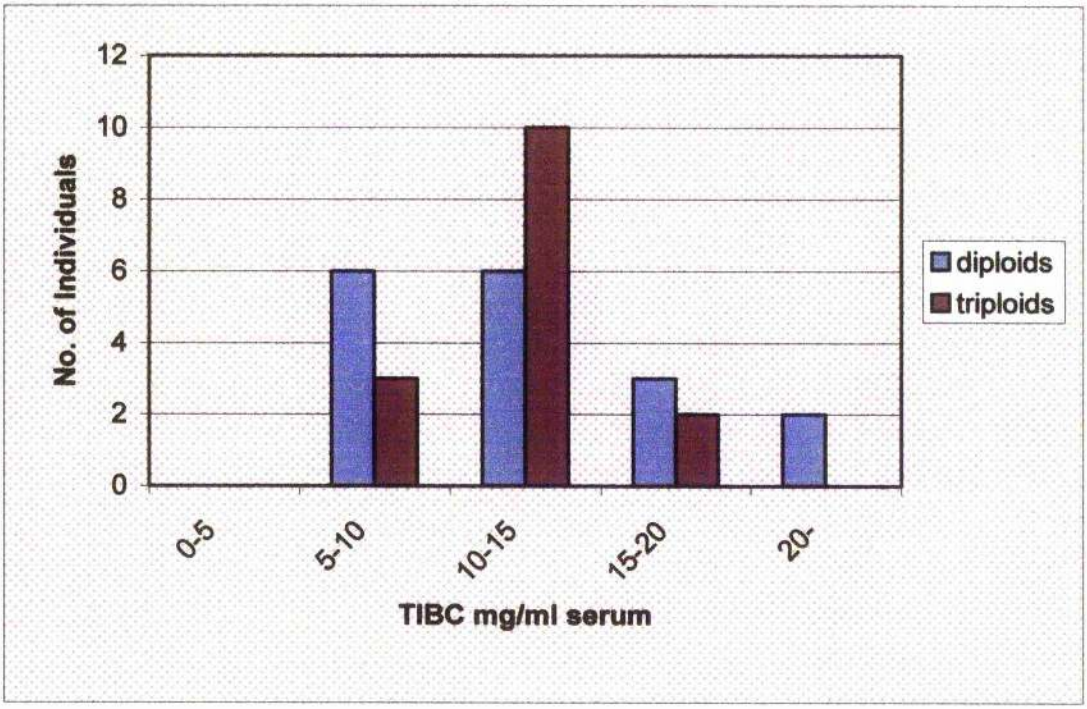


Figure 3.4 Experiment B: Frequency distribution of TIBC in diploid and triploid, all-female Atlantic salmon.

Figures 3.3 and 3.4 show the distributions of alternative pathway complement activity (ACH50) and TIBC in the diploid and triploid groups. Although the mean ACH50 does not differ and there is no statistical difference between the groups, the highest and lowest responding fish are triploids. However, this pattern was not the same for the other non-specific, humoral parameters (for example TIBC).

3.4 Discussion

3.4.1 Serum lysozyme

Serum lysozyme was not detected in measurable quantities in the experimental fish (diploids and triploids) used in the present study. It may be that the assay is simply not able to detect low levels of lysozyme. There are technological restraints including that the microplate reader has an error of 0.001 to 0.002 OD that can occur between readings. This was sometimes noted in the blanks. Combined with the miniturisation of the assay this makes it difficult to detect levels which would cause a decrease in OD of only 0.002 (this is 80 lysozyme units/ml in neat serum).

Lysozyme has been detected in the sera of various fish species, including plaice (Fletcher and White, 1973), rainbow trout (Grinde *et al.*, 1988), paddlefish (Holloway *et al.*, 1993), Walleye (Holloway, *et al.*, 1993) and sea trout (Muona and Soivio, 1992). It has also been detected in Atlantic salmon (Fevolden *et al.*, 1994; Grinde, *et al.*, 1988; Lie *et al.*, 1989; Muona and Soivio, 1992). It was concluded that serum lysozyme levels in this study were immeasurably low.

Muona and Soivio (1992) monitored plasma lysozyme levels of Atlantic salmon over several months. They used a turbidimetric assay, monitoring the lysis of *Micrococcus lysodeikticus*, similar to that used in this study. Their results showed that salmon had highest lysozyme levels in the winter which then decreased to a minimum in spring. The changes seemed to be related to water temperature and physiological changes associated with smolting. Both of these factors may be stress factors.

Lysozyme activity has been found to vary with stress or infection. Although most studies report increases in lysozyme activity (Fevolden, *et al.*, 1994; Moeyner *et al.*, 1993), one reports a decrease (Mock and Peters, 1990). As mentioned above, lysozyme activity can vary according to environmental factors such as water temperature and bacterial presence (Fletcher and White, 1976). Grinde *et al.*, (1988) found that lysozyme levels can vary even to very small changes in the environment.

Lysozyme has been shown to occur in high levels in white blood cells (Fletcher and White, 1973). The high presence of lysozyme in white blood cells may be the reason for the reported increase in lysozyme activity during immunisation and in diseased or stressed fish, since leucocytes lyse in response to these, thereby releasing internal lysozyme.

In this study, every attempt was made to minimise exposure of the fish to potential stress factors. This was to avoid the possibility that triploid fish may be more 'susceptible' to stress and therefore avoiding potentially differential results caused by their theoretical differential response to stress. Also, these fish were sampled in March 1995 (Experiment A) and February 1996 (Experiment B), ie. they were sampled during spring. The combination of the fish not being stressed and being sampled in spring may explain why lysozyme was not detected in this study.

Grinde *et al.* (1988) and Lie *et al.* (1989) reported lysozyme levels in Atlantic salmon which were measured in kidney homogenates. Lysozyme activity of most fish species is higher in the kidney than in serum or plasma (Grinde, *et al.*, 1988; Lie, *et al.*, 1989). For future studies it may therefore be advisable to measure kidney lysozyme activity rather than that of serum.

3.4.2 Complement Activity

Complement activity initiated by either the classical or alternative pathways, did not significantly differ between diploids and triploids in these experiments. This concurs with other investigations of complement activity in induced triploids. Levels of alternative pathway complement activity were found to be higher in triploid ayu in

comparison to diploids, but not significantly (Kusuda *et al.*, 1991) and Yamamoto and Iida (1995b) found no significant differences in alternative pathway complement or serum bactericidal activity of triploid rainbow trout.

3.4.3 Serum antiproteinase activity

Total anti-trypsin activity of Atlantic Salmon serum did not appear to differ between diploid and triploid fish. However, triploids were deficient in α_2-macroglobulin compared to their diploid siblings. This has not been measured in other triploid fish.

Upon invasion of a host, many pathogens release enzymes (including proteinases) that aid penetration of and release nutrients from the host's tissues. The host in contrast has a repertoire of enzyme inhibitors (including antiproteinases) which act to prevent autodigestion and also to combat these enzymes released by pathogens. Antiproteinases can inhibit a wide range of proteinase enzymes but this assay was designed to determine anti-trypsin activity only. The 2 main trypsin inhibitors present in fish serum are α_2-macroglobulin and α_1-antiproteinase, of which α_2 macroglobulin has the most non-specific nature (Bowden *et al.*, 1997). Total-anti trypsin activity described by this assay is measurement of the activity of all serum inhibitors which act against trypsin.

Since antiproteinases inhibit the function of otherwise potentially hazardous enzymes secreted by invading organisms (Alexander and Ingram, 1992; Ellis, 1981), one outcome of this difference in antiproteinase levels may be varying susceptibilities to different strains of pathogens depending on the types of extracellular proteases released by the invading organism. Due to the wide range of immune parameters present in fish, however, it is unlikely that the deficiency in α_2-macroglobulin alone will render the triploid immune system less efficient than that of a diploid fish.

3.4.4 Serum iron with-holding properties

Serum iron levels and iron-binding capacities are traditionally expressed as mg/dl, for humans, mammals and fish. However, in order to facilitate comparison with other factors, the present results have been expressed as mg/ml. For comparison to the literature, the reported data have been converted to mg/ml.

Transferrin has been identified in fish including Atlantic salmon (Roed, *et al.*, 1995). It exhibits considerable genetic polymorphism. In Coho salmon, three electrophoretically different forms of transferrin have been demonstrated (Utter *et al.*, 1970). The iron-binding properties of fish serum are also well documented. Hershberger and Pratschner (1981) studied iron-binding capacities of 6 transferrin phenotypes in Coho salmon and found the mean to be 0.85mg/ml for total serum iron, with total iron-binding capacity (TIBC) of 3.65mg/ml, unsaturated iron-binding capacity (UIBC) of 2.8mg/ml and percentage saturation of transferrin of 23%. Differences among phenotypes were insignificant. The percentage iron saturation of rainbow trout serum transferrin is 19.8-38.5% (Congleton and Wagner, 1991). Ravndal *et al.* (1994) found total serum iron levels of 1.51-2.1mg/ml for full sib family Atlantic salmon, and 1.67-1.91mg/ml for half sib salmon.

The values found in this study were higher than those reported in the literature for Atlantic salmon. Total serum iron is almost twice that reported by Ravndal *et al.*, (Ravndal, *et al.*, 1994). Hershberger and Pratschner (Hershberger and Pratschner, 1981) found that TIBC of Coho salmon varied according to rearing habitat. TIBC of pen reared fish was higher than that of angler-caught fish. This may suggest that iron with-holding parameters can vary with diet and/or physiological status. Malnutrition has been shown to reduce synthesis of transferrin (Hershberger and Pratschner, 1981) and thereby affect TI and TIBC.

Serum iron with-holding activity has not been measured previously in triploid fish.

4: Kinetics of responsiveness of some non-specific, humoral immune parameters in diploid and triploid Atlantic salmon.

4.1 Introduction

In the previous chapter the constitutive levels of various non-specific defence factors were measured and diploids and triploids had little difference. Since one of the major differences between diploid and triploid fish is that triploids have larger and fewer cells it was felt that this may have effects on the kinetics of changes in these levels following an induced disturbance. This chapter investigates the kinetics of non-specific humoral immunity in response to injection of lipopolysaccharide. This was chosen as a means of inducing a response to mimic that which might occur during a Gram-negative bacterial infection.

The kinetics of two components of the non-specific humoral immune response were studied; the iron with-holding system and alternative pathway complement activity.

4.1.1 Iron with-holding system

Iron with-holding is a system which attempts to deny invading organisms and neoplastic cells access to iron which is essential for growth. It has both constitutive and induced components (described in Chapter 3.1); the former provides a series of defenses which are constantly present whereas the latter is an induced system of removing iron from the areas where it could be acquired by pathogens.

In mammals, induced components of the system are activated by cytokines such as interleukins 1 and 6, and tumour necrosis factor (TNF) (Weinberg, 1992). Once activated the induced components cause the hypoferraemic response. Congleton and Wagner (1991) have shown that fish exhibit an acute phase hypoferraemic response. The mechanism by which the hypoferraemic response is initiated in fish is unknown but in mammals it is mediated mostly by interleukin-1 (IL-1). During an acute phase response elevated IL-1 levels occur which mobilize neutrophils to sites of invasion and stimulate the release of lactoferrin by neutrophils (Klempner *et al.*, 1978). The lactoferrin removes iron from serum transferrin forming lactoferrin-iron complexes

that are rapidly cleared from circulation. This lowers the plasma or serum iron levels and diverts the iron to the liver (Weinberg, 1984; Weinberg, 1992). Lactoferrin has also been reported to affect other immune functions in mammals, including cytokine production, complement activation, lymphocyte proliferation and bacterial killing (Brock, 1995; Weinberg, 1984). Other induced components of the system suppress intestinal assimilation of iron and cause retention of iron by macrophages. These responses are described in more detail in Chapter 3.1.

However, to the author's knowledge there is no evidence in the published literature of lactoferrin in fish.

This initation of the iron-with-holding system in response to infection, inflammation or injury is part of a multi-faceted response often referred to as the acute phase response. Many of the acute phase responses in mammals are activated in a similar way to the induced iron-with-holding components; by soluble mediators such as cytokines. However, it is not fully understood how these responses are initiated in fish.

A number of cytokines have an involvement in inflammatory responses in mammals, including tumor necrosis factor α (TNFα), interleukin 1 (IL-1), IL-6 and a variety of chemokines. TNFα is the principal mediator of the host response to Gram negative bacteria and itself prompts the release of other cytokines (Secombes *et al.*, 1996). IL-1, as already mentioned, prompts the hypoferraemic response in mammals. It has been shown that fish or fish cells can produce a variety of cytokine-like substances including IL-1, IL-2, interferons and TNF (Manning and Nakanishi, 1996). There is also tentative evidence of IL-3, IL-4, IL-6 and other cytokines in fish.

IL-1 and TNF are mainly produced by macrophages and monocytes in mammals (Manning and Nakanishi, 1996) and in fish (Manning and Nakanishi, 1996), although they can be secreted by other cell types. Certain components of microbial cell walls act as stimulants for their release by mammalian macrophages and

monocytes, including lipopolysaccharide (LPS). LPS has been extensively used to induce acute phase responses in mammals. While LPS appears to have no acute toxicity in fish (Wedemeyer *et al.*, 1968), there is evidence that it can induce certain acute phase responses in fish including liver glycogenolysis (Wedemeyer, *et al.*, 1968) and hypoferraemia (Congleton and Wagner, 1991).

4.1.2 Alternative pathway complement activity

The complement system is an integral part of the immune response of fish as with other higher vertebrates and is described in previous chapters. In the alternative complement pathway (ACP), C3 is activated directly in the presence of factors B and D. This results is the activation of a cascade of complement components (C5, C6, C7, C8 and C9). C3 can be activated by a variety of substances including the lipopolysaccharide (LPS) of Gram-negative bacteria. Fish complement has bactericidal activity against Gram-negative but not usually against virulent Gram-negative or Gram-positive bacteria. This is possibly, at least in part, due to activation of the ACP by LPS on the cell wall. Lamas and Ellis (1994) have shown that *Aeromonas salmonicida* activates ACP and causes depletion of serum complement, *in vitro*.

4.2 Materials and Methods

4.2.1 Experimental Design

Ten diploid and 10 triploid Atlantic salmon (mixed sex) were injected with 1000μg lipopolysaccharide ((LPS) B5:55 *E. coli*, Sigma) per kg body weight. Also, 10 diploids and 10 triploids were injected with 0.9% saline as controls. The average weight of the fish was 2.1kg. The fish were individually marked with fly-tags at the same time as treatment, and kept in flow-through seawater at ambient temperature (average 12°C) for 20 days. Each individual was bled at days 0, 1,2,3,5,11 and 19 after injection, and the serum extracted according to the method described below. Total iron content (TI), unsaturated iron binding capacity (UIBC) and alternative pathway complement activity (ACH_{50}) were measured in the treated and control fish

according to the methods described below. At the end of the trial, ploidy was confirmed by inspection of the gonads and examination of blood smears.

4.2.2 Sample collection

One ml of blood was collected from the caudal vein of fish anaesthetised with metamidate, using needle and 1ml syringe. Blood was placed into a centrifuge tube and allowed to clot at room temperature. The clotted blood was centrifuged and serum collected. Serum was transported to the laboratory on dry ice and stored at -80°C until analysed.

4.2.3 Serum assays

Protein assay

Serum protein was measured using commercially available reagents (BCA protein assay Kit, Pierce). Briefly, 200µl of working reagent was added to bovine serum albumin standards, blanks (distilled water) and diluted serum in a 96 well microtitre plate. Serum was diluted 1 in 25, 1 in 50 and 1 in 100 in distilled water. The microtitre plate was incubated at 37°C for 30 min and absorbance measured at 570nm. The protein concentration was calculated for each dilution from a standard curve, the mean of the 3 dilutions was taken for each sample and the results reported as protein mg/ml of serum.

Alternative Pathway Complement Activity.

This assay was adapted from Yano (1992) and miniaturised for use in microtitre plates as described in Chapter 3.2.3.

Total iron content and unsaturated iron binding capacity

These parameters were determined using commercially available reagents (Sigma No. 565) as described in Chapter 3.2.3.

4.2.4 Statistical analysis

Graphs showing individual data were produced and mean profiles superimposed to show mean trends over time.

TIBC and percentage saturation are derived from TI and UIBC. Therefore, TIBC and percentage saturation were not analysed statistically. The distributions for TI, UIBC, ACH50 and serum protein concentration were studied using histograms and then transformed as required to produce normal distributions. Multivariate analysis of variance (MANOVA) was performed to determine whether there were any ploidy or treatment differences. Ante-dependency tests were performed to confirm the conclusions drawn from the multivariate analysis.

4.3 Results

The data are shown in Tables 4.1-4.6. While each group comprised 10 fish, there were not always 10 samples available for each group at each sample point. Each fish has been considered separately to avoid problems caused by individual variation. From this, patterns of changes in the parameters for each treatment group have been analysed

4.3.1 Protein

Figure 4.1 shows the serum protein concentration (mg/ml) for each individual in each treatment group over the course of the experiment. There is variation evident between individuals and also within the data for an individual fish.

From ante-dependency and MANOVA tests, at Day 0, the serum protein concentration of triploids is higher than that of diploids ($p=0.001$ from MANOVA). However, this diploid/ triploid difference does not persist and is not evident at any other time point.

From the graphs it is evident that serum protein decreases in all groups over time.

4.3.2 Alternative pathway complement activity (ACH50)

Figure 4.2 shows the ACH50 for each individual in each treatment group over the course of the experiment. There was high individual variation, even though there was variation within the data for an individual fish.

There were no significant differences between diploids and triploids at Day 0.

MANOVA showed that there was a significant difference between treatments ($p<0.001$). Ante-dependency tests show that the treatment effect occurs mainly on Days 2 and 3 but also a smaller effect at Day 5. From the graphs, it can be seen that the differences between treatments manifest themselves as a dramatic decline in ACH50 at Days 2 and 3 with partial recovery at Day 5 for the groups treated with LPS. Those groups treated with saline do not show any changes over time.

MANOVA analysis suggests that there was also marginal evidence of differences between the ploidy groups ($p=0.04$). Ante-dependency analysis shows that this difference occurs at Day 5. When considered in parallel with the graphs this suggests that diploids treated with LPS recover to baseline levels more quickly than triploids.

4.3.3 Total serum iron content

Figure 4.3 shows the serum iron content (mg/ml) for each individual in each treatment group over the course of the experiment. There was high individual variation.

There were no significant differences between diploids and triploids at Day 0.

MANOVA suggests that there is a treatment effect ($p=0.051$). Ante-dependency tests show that this treatment effect occurs at Days 2 and 3. Both saline and LPS treated fish showed decreases in TI by Days 2 and 3 with recovery at between Days 11 and 15. The treatment effects described above and graphical evidence show that

those fish treated with LPS had lower levels of TI at Days 2 and 3 than fish treated with saline.

A weak ploidy effect was shown by MANOVA ($p=0.72$). Ante-dependency tests show that this ploidy effect occurs at Day 2. When considered with the graphical evidence, this suggests that diploids (but only those treated with LPS) have a lower TI at Day 2 than triploids.

4.3.4 Unsaturated iron binding capacity (UIBC)

Figure 4.4 shows the UIBC (mg/ml) for each individual in each treatment group over the course of the experiment.

MANOVA shows that there are no significant ploidy or treatment effects. Ante-dependency tests suggest that there is weak evidence of treatment effect at Day 15 and this focused on the LPS injected diploid group.

The graphs show that there is a slight decrease in UIBC in all groups towards the end of the experiment.

4.3.5 Total iron binding capacity (TIBC)

Figure 4.5 shows the TIBC (mg/ml) for each individual in each treatment group over the course of the experiment.

Statistical analyses were not done since this parameter is dependent on TI and UIBC. TI is small in comparison with UIBC and therefore TIBC will vary according to changes in UIBC. The graphs show that TIBC follows the same pattern as UIBC with a slight decrease in all groups towards the end of the experiment.

Table 4.1 Serum protein concentrations (mg/ml), as monitored over 20 days, of individual diploid and triploid Atlantic salmon injected with either 0.9% NaCl or lipopolysaccharide.

Diploids injected with 0.9% NaCl

FishDay	0	1	2	3	5	11	15	19
4401	34.75	38.90	29.10	28.10	21.20	40.10	29.82	26.56
4402	33.60	34.40	35.10	23.74	31.25	59.55	20.90	34.20
4403	23.97	39.45	38.43	61.90	22.40	32.60	36.30	15.00
4404	27.20	24.30	31.30	33.13	53.50	33.67	33.20	26.75
4405		32.23	41.40	36.75	47.73	33.80	20.90	30.63
4406	31.80	36.90	25.45	36.35	51.50	39.12	46.10	29.40
4407		52.20	29.43	24.40	44.24	34.40	32.70	23.56
4408	38.55	26.72	25.11	20.16	30.76	39.00	42.91	21.43
4409	27.17	28.43	24.00	23.03	38.11	32.90	33.78	24.80
4410	28.32		31.00	39.90	56.23	26.96	26.43	19.10
Mean	30.67	34.77	31.03	32.72	39.69	37.21	32.30	25.14

Triploids injected with 0.9% NaCl

FishDay	0	1	2	3	5	11	15	19
4421	45.40	38.65	33.70	35.23	42.20	28.33	20.13	19.30
4422	54.50	21.33	38.05	25.57	49.76	34.20	19.87	25.33
4423	38.90	45.97	23.87	38.17	40.00	43.70	38.70	17.17
4424	51.60	35.10	33.85	38.90	25.33	31.00	26.50	16.70
4425	41.50	34.70	37.20	43.85	35.37	21.50	20.00	21.70
4426	37.00	26.07	36.80	55.90	34.60	42.10	36.80	23.80
4427	51.60	83.40	36.30		33.13	40.60	42.60	17.83
4428	50.01	24.70	45.00	39.40	38.60	71.20	22.13	19.25
4429	37.70	29.53		28.83	43.90	31.50	33.40	21.00
4430		24.17		59.20	25.97	31.13	33.60	18.87
Mean	45.40	36.36	35.60	40.43	36.89	37.56	29.57	20.10

TRIPLOIDS

DIPLOIDS

Diploids injected with lipopolysaccharide

FishDay	0	1	2	3	5	11	15	19
4411	24.57	33.00	38.23	41.20	23.90	32.23	36.00	25.80
4412	34.16	38.88	42.00			33.76	71.80	15.63
4413	26.36	18.90	28.60	36.70		26.77	18.03	28.25
4414	22.43	30.63	19.80	39.07	33.42	37.60	42.45	23.20
4415	36.70	26.70	32.67	43.20	22.50	32.57	38.30	56.45
4416	29.70	19.86	30.45	48.00	34.51	33.55	32.10	32.70
4417	23.10	30.53		32.47	27.06			
4418	33.20	23.63	34.85	44.56	42.50	23.66	36.70	19.90
4419	41.25	25.30	34.40	58.90	36.45	37.20	71.70	42.85
4420	52.30		27.20	45.30		35.67	41.90	38.20
Mean	32.38	27.49	32.00	43.27	31.45	32.56	43.22	31.40

Triploids injected with lipopolysaccharide

FishDay	0	1	2	3	5	11	15	19
4581	36.00	36.30	41.86	83.30	33.96	38.40	22.40	32.00
4582	54.45	32.70	39.00	41.30	34.50	36.10		18.60
4583	55.90	28.06	38.12	37.70	35.45	39.40	23.90	28.86
4584	46.55	49.90	43.70	22.58	28.40	32.85	22.40	36.70
4585	37.30	53.10	23.50	36.30		61.00	34.50	31.56
4586	57.85	24.05	31.07	34.20		22.95	36.40	24.77
4587	64.70	29.60	24.20	29.54	34.44	33.46	49.90	23.70
4588	35.70	14.80	31.90	42.30	27.86		32.00	25.80
4589	32.45	41.30	27.00	32.54	29.05	33.12		27.30
4590	36.60	60.40	54.95	36.86	39.50	24.37	28.40	30.63
Mean	45.95	37.02	35.43	39.67	32.85	35.72	31.24	27.36

Table 4.2 Alternative pathway complement activity (ACH50 units/ml), as monitored over 20 days, of individual diploid and triploid Atlantic salmon injected with either 0.9% NaCl or lipopolysaccharide.

Diploids injected with 0.9% NaCl

Fish/Day	0	1	2	3	5	11	15	19
4401	144.37	210.43	207.02	204.86	155.55	124.71	199.53	192.73
4402	165.70	208.47	237.15	155.48	139.47	279.27	259.98	117.37
4403	107.34	260.24	282.13	110.43	146.85	234.37	180.11	240.99
4404	134.72	146.47	103.70	135.79	98.52	105.29	153.85	90.14
4405	84.84	67.19	75.99	40.07	70.47	66.83	82.54	88.52
4406	79.03	97.76	43.55	58.18	107.54	109.28	138.18	83.75
4407	101.93	130.26	80.37	87.99	92.89	79.55	54.55	54.89
4408	136.69	69.37	61.87	73.85	82.64	96.30		52.33
4409	214.43	165.15	152.76	109.17	151.94	175.94	134.27	158.42
4410	80.23	53.23	64.77	72.26	99.85	70.09		
Mean	124.93	142.86	128.93	104.81	120.56	134.16	150.50	120.02

Diploids injected with lipopolysaccharide

Fish/Day	0	1	2	3	5	11	15	19
4411	131.31	47.63	23.73	3.59	53.90	66.51		60.12
4412		136.59	18.94	8.20		133.61	124.26	195.63
4413	77.33	113.29	26.28	20.25	69.08	114.14		199.33
4414	132.21	89.63	18.66	70.77		88.27	74.79	147.47
4415	78.87	196.08	10.48	8.58	115.51	200.79	210.43	122.08
4416	121.88	64.83	18.13	4.74	235.73	131.30		85.92
4417	106.73	121.68	57.60	1.85	116.60	102.08	90.86	
4418	107.53	106.73	53.99	6.71	135.48	229.97	167.10	78.70
4419	43.58	65.00	38.48	9.62	67.27		134.62	32.36
4420	134.92	171.67	30.57	6.87	104.69	118.98	148.63	
Mean	103.80	109.31	29.69	14.12	112.28	131.52	135.51	115.20

TRIPLOIDS

Triploids injected with 0.9% NaCl

Fish/Day	0	1	2	3	5	11	15	19
4421	97.39	139.02	117.44	180.85	154.11	110.13	135.09	65.00
4422	79.25	135.62	132.10	201.87	99.22	107.97	54.16	73.93
4423	199.53	292.51	271.72	278.61	203.46	194.89	154.04	
4424	135.09	137.83	159.00		88.16	77.88	71.80	53.50
4425	153.85	165.99	126.54	170.74	105.55	251.44	160.60	148.23
4576	103.61	96.23	60.77	99.26	44.51	75.31		188.39
4577	51.54	119.61	167.10	103.59	122.60	135.95	150.85	
4578	231.35	71.09	94.95	135.22	134.27	44.27		83.57
4579	79.39	67.77	145.56	174.59	64.32	99.86	89.82	90.75
4580	95.72	73.80	64.88	36.62	100.81	54.04		
Mean	122.67	129.93	134.01	153.48	111.70	115.17	117.91	99.05

Triploids injected with lipopolysaccharide

Fish/Day	0	1	2	3	5	11	15	19
4581	136.37	139.52	14.15	12.86	18.61	56.52	159.89	219.55
4582	62.43	151.94	32.69	14.07	76.91	250.52	144.27	71.95
4583	115.88	121.94	17.46	5.24	34.24	130.58	197.48	118.70
4584	187.36	140.57	39.32	14.20	22.95	98.68	76.52	84.68
4585	162.12	36.26	75.11	19.04	82.86	84.68		
4586	77.71	206.48	78.59	15.48	34.73	111.01	71.49	90.73
4587	198.78	101.41	98.72	12.76	52.73	82.79	215.78	269.96
4588	101.27	107.98	80.96	14.04	80.69	197.48	132.10	82.79
4589	160.65	129.67	48.71	17.09	44.17	114.52	131.30	98.84
4590	83.38	111.23	75.55	19.47	39.57	76.50	99.22	97.02
Mean	130.60	129.90	56.13	14.43	48.77	120.33	136.45	131.28

Table 4.3 Total serum iron content (mg/ml), as monitored over 20 days, of individual diploid and triploid Atlantic salmon injected with either 0.9% NaCl or lipopolysaccharide.

Diploids injected with 0.9% NaCl

Fish/Day	0	1	2	3	5	11	15	19
4401	0.21	0.24	0.17	0.12	0.07	0.09	0.44	0.34
4402	0.32	0.18	0.13	0.11	0.22	0.51	0.38	0.26
4403	0.28		0.22	0.14	0.05	0.10	0.09	0.23
4404	0.57	0.74	0.45	0.08	0.05	0.51	0.48	0.17
4405	0.22	0.11	0.15	0.08	0.14	0.22	0.49	0.33
4406	0.74	0.81	0.19	0.06	0.12	0.09	0.38	0.43
4407	0.34	0.28	0.11	0.06	0.21	0.43	0.57	0.44
4408	0.39	0.28	0.18	0.11	0.01	0.22	0.34	0.34
4409	0.26	0.28	0.23	0.11	0.06	0.12	0.17	0.22
4410	0.46	0.34	0.22	0.14	0.02	0.07	0.39	0.57
Mean	0.38	0.34	0.21	0.10	0.10	0.24	0.37	0.34

Triploids injected with 0.9% NaCl

Fish/Day	0	1	2	3	5	11	15	19
4421	0.24	0.20	0.12	0.17	0.05	0.29	0.40	0.34
4422	0.41	0.50	0.40	0.11	0.15		0.37	0.34
4423	0.39	0.43	0.24	0.15	0.12	0.20	0.33	
4424	0.33	0.37	0.24	0.18	0.08	0.14	0.30	0.43
4425	0.33	0.39	0.21	0.14	0.16	0.30	0.53	0.41
4426	0.44	0.23	0.22	0.14	0.07	0.20	0.39	0.20
4427	0.34	0.36	0.26	0.06	0.05	0.24	0.47	0.32
4428	0.62	0.51	0.53	0.33	0.09	0.21	0.53	0.73
4429	0.23	0.12	0.21	0.28	0.11	0.17	0.27	0.38
4430	0.48	0.49	0.17	0.11	0.18	0.32	0.39	0.43
Mean	0.38	0.36	0.26	0.17	0.11	0.23	0.40	0.40

TRIPLOIDS

DIPLOIDS

Diploids injected with lipopolysaccharide

Fish/Day	0	1	2	3	5	11	15	19
4411	0.28	0.13	0.01	0.09	0.19	0.21	0.32	0.27
4412	0.29	0.22		0.06	0.06	0.12	0.34	0.41
4413	0.30	0.32	0.11	0.06	0.19	0.27	0.34	0.28
4414	0.60	0.17	0.01	0.02	0.09	0.11	0.39	0.74
4415	0.26	0.29	0.12	0.04	0.15	0.21	0.36	0.41
4416	0.24	0.14	0.04	0.19	0.25	0.31	0.34	0.32
4417	0.28	0.15	0.08	0.09	0.16	0.27	0.36	0.31
4418	0.38	0.27	0.03	0.09	0.17	0.14		0.44
4419	0.39	0.21	0.09	0.06	0.18	0.32	0.52	0.46
4420	0.32	0.12	0.07	0.02	0.14	0.21	0.32	0.30
Mean	0.33	0.20	0.06	0.07	0.16	0.22	0.37	0.39

Triploids injected with lipopolysaccharide

Fish/Day	0	1	2	3	5	11	15	19
4581	0.22	0.14	0.09	0.08	0.17	0.21	0.32	0.11
4582	0.29	0.32	0.14	0.12	0.06	0.16	0.22	0.31
4583	0.38	0.45	0.17	0.02	0.08	0.13	0.21	0.36
4584	0.46	0.44	0.07	0.01	0.10	0.14	0.25	0.18
4585	0.36	0.50	0.39	0.07	0.09	0.13	0.47	0.29
4586	0.34		0.16	0.05	0.10	0.16	0.17	0.21
4587	0.23	0.34	0.29	0.07	0.08	0.47	0.51	0.35
4588	0.51	0.19	0.16	0.06	0.06	0.11	0.47	0.39
4589	0.43	0.17	0.12	0.09	0.11	0.21	0.37	0.28
4590	0.23	0.24	0.14	0.03	0.05	0.12	0.19	0.22
Mean	0.34	0.31	0.17	0.06	0.09	0.18	0.32	0.27

Table 4.4 Unsaturated iron binding capacity (mg/ml), as monitored over 20 days, of individual diploid and triploid Atlantic salmon injected with either 0.9% NaCl or lipopolysaccharide.

Diploids injected with 0.9% NaCl

FishDay	0	1	2	3	5	11	15	19
4401	4.60	3.81	4.67	4.76	4.43	4.42	4.02	3.67
4402	3.68	2.66	3.39	4.51	3.78	3.48	3.79	3.79
4403	3.81	3.19	3.25	3.36	4.08	4.09	3.53	
4404	4.61	4.51	3.26	3.73	3.76	4.19		4.19
4405	4.49	3.42	3.50	4.15	4.14	3.04	3.74	3.06
4406	4.94	3.24	4.69		4.26	3.59	4.24	3.01
4407	3.49	4.29		3.83		4.21	4.25	3.80
4408	4.25	4.23	4.11	4.48	3.75		3.91	2.85
4409	4.08	3.76	3.82	4.02		3.00	3.14	3.15
4410	4.23	3.38	4.03	3.21	2.79	3.61	3.96	1.71
Mean	4.24	3.65	3.86	4.02	3.87	3.74	3.84	3.25

Triploids injected with 0.9% NaCl

FishDay	0	1	2	3	5	11	15	19
4421	4.16	3.20	3.63	2.96	3.12	3.49	2.39	2.08
4422	3.27	4.05	4.13	4.62	4.81	3.84	3.92	2.94
4423	3.82		3.25	2.95	3.80	4.40	4.09	3.97
4424	4.67	4.68	4.96	4.74	4.40	4.20	3.79	3.44
4425	3.39	3.25	4.41	4.12	4.30		4.12	2.59
4426	3.24	4.94	3.67	4.66	4.31	4.34	3.89	3.59
4427	3.39	3.67	3.52	3.87	4.08	3.78	3.95	3.25
4428	3.04	2.85	2.94	2.97	2.72	3.36	2.37	2.33
4579	3.75	4.09	4.46	3.78	4.32	3.99	4.11	4.21
4580	4.96	4.52	3.47	3.49	3.91	4.39	4.24	4.68
Mean	3.77	3.94	3.86	3.82	3.98	3.97	3.69	3.31

TRIPLOIDS

DIPLOIDS

Diploids injected with lipopolysaccharide

FishDay	0	1	2	3	5	11	15	19
4411	3.46	3.96	4.38	4.21	4.19	3.43		3.24
4412	4.60	3.97	4.66	3.88	3.58	3.72		
4413	3.98	4.04	4.19	4.86	4.53	3.88	2.81	
4414	3.98	3.53	3.12	4.41	4.08	3.14		2.89
4415	3.62	3.65	4.61	3.75	4.56	3.82		3.06
4416	3.67	4.93	4.16	4.59	4.58	4.06	2.57	2.38
4417	3.81	3.76	4.08	4.42	4.22	3.14	3.33	2.15
4418	4.46	4.09	3.96	4.56	4.03	3.02	2.34	1.22
4419	3.64	3.59	4.45	3.27	3.43	3.24	2.94	2.52
4420	3.92	4.16	4.11	3.94	3.87	3.16	2.01	1.97
Mean	3.92	3.97	4.17	4.19	4.11	3.47	2.75	2.39

Triploids injected with lipopolysaccharide

FishDay	0	1	2	3	5	11	15	19
4581	4.10	4.24	4.24	4.02	4.42	3.71	3.89	2.94
4582	3.84	3.20	3.25	3.62	3.03	2.95	2.74	
4583	4.43	4.39	4.46	4.07	4.18	4.29	4.10	3.37
4584	3.10	4.25	3.45	2.54	3.65	4.16	2.89	2.86
4585	3.78	3.20	4.08	4.56	4.67	2.37	2.67	2.16
4586	3.48	3.83	4.26	4.51	3.89	3.56	3.69	3.25
4587	3.95	4.83	4.92	4.58	4.27	4.18	3.44	
4588	3.33	3.48	3.58	3.98	3.77	4.04		3.36
4589	3.70	4.08	3.14	4.22	3.84	2.92	3.15	
4590	3.66	3.69	4.59	3.87	3.64	4.55	3.62	3.18
Mean	3.74	3.92	4.00	4.00	3.94	3.67	3.38	3.09

Table 4.5 Total iron binding capacity (mg/ml), as monitored over 20 days, of individual diploid and triploid Atlantic salmon injected with either 0.9% NaCl or lipopolysaccharide.

Diploids injected with 0.9% NaCl

Fish/Day	0	1	2	3	5	11	15	19
4401	5.01	4.05	4.84	4.88	4.50	4.51	4.46	4.01
4402	4.00	2.84	3.52	4.62	4.00	3.99	4.17	4.05
4403	4.09		3.47	3.50	4.13	4.19	3.62	
4404	5.18	5.25	3.71	3.81	3.81	4.70		4.36
4405	4.71	3.53	3.65	4.23	4.28	3.26	4.23	3.39
4406	5.68	3.85	4.88		4.38	3.68	4.62	3.44
4407	3.83	4.57		3.99		4.64	4.82	4.24
4408	4.64	4.49	4.29	4.59	3.76		4.25	3.19
4409	4.34	4.04	4.05	4.13		3.12	3.31	3.37
4410	4.69	3.72	4.25	3.35	2.81	3.68	4.35	2.28
Mean	4.62	4.04	4.07	4.12	3.96	3.97	4.20	3.59

Diploids injected with lipopolysaccharide

Fish/Day	0	1	2	3	5	11	15	19
4411	3.74	4.09	4.37	4.30	4.38	3.64	3.56	3.06
4412	4.89	4.19		3.94	3.64	3.84		
4413	4.28	4.36	4.30	4.92	4.72	4.15		2.78
4414	4.59	3.70	3.13	4.43	4.17	3.25	3.20	3.63
4415	3.88	3.94	4.73	3.79	4.71	4.13		3.47
4416	3.91	5.07	4.20	4.78	4.63	4.37	2.91	2.70
4417	4.10	3.91	4.16	4.51	4.38	3.41	3.69	2.46
4418	4.86	4.36	3.89	4.65	4.20	3.16		1.66
4419	4.03	3.80	4.54	3.33	3.62	3.56	3.46	2.98
4420	4.24	4.28	4.16	3.96	4.01	3.37	2.33	2.27
Mean	4.25	4.17	4.18	4.26	4.27	3.89	3.19	2.78

DIPLOIDS

TRIPLOIDS

Triploids injected with 0.9% NaCl

Fish/Day	0	1	2	3	5	11	15	19
4421	4.40	3.40	3.75	3.15	3.17	3.78	2.79	2.42
4422	3.68	4.55	4.53	4.73	4.96		4.29	3.28
4423	4.21		3.49	3.10	3.92	4.60	4.42	
4424	5.00	5.05	5.20	4.92	4.48	4.34	4.09	3.87
4425	3.72	3.64	4.62	4.26	4.46		4.65	3.00
4576	3.68	5.17	4.09	4.80	4.38	4.54	4.28	3.79
4577	3.73	4.23	3.78	3.83	4.13	4.00	4.42	3.57
4578	3.66	3.36	3.47	3.30	2.81	3.57	2.90	3.06
4579	3.98	4.21	4.67	4.06	4.43	4.16	4.38	4.59
4580	5.42	5.01	3.64	3.60	4.09	4.71	4.63	5.11
Mean	4.15	4.29	4.12	3.99	4.08	4.21	4.08	3.63

Triploids injected with lipopolysaccharide

Fish/Day	0	1	2	3	5	11	15	19
4581	4.32	4.38	4.33	4.10	4.59	3.92	4.21	3.95
4582	4.13	3.52	3.39	3.74	3.09	3.11	2.96	
4583	4.81	4.84	4.63	4.09	4.26	4.42	4.31	4.23
4584	3.56	4.69	3.52	2.55	3.75	4.30	3.14	3.06
4585	4.14	3.70	4.47	4.63	4.76	2.50	3.14	2.45
4586	3.82		4.42	4.56	3.99	3.72	3.86	3.46
4587	4.18	5.17	5.21	4.55	4.35	4.65	3.95	
4588	3.84	3.67	3.74	4.04	3.82	4.15		3.75
4589	4.13	4.25	3.26	4.31	3.85	3.13	3.52	
4590	3.89	3.93	4.73	3.90	3.69	4.67	4.01	3.40
Mean	4.08	4.24	4.17	4.06	4.03	3.86	3.68	3.34

Table 4.6 Percentage transferrin saturation (%), as monitored over 20 days, of individual diploid and triploid Atlantic salmon injected with either 0.9% NaCl or lipopolysaccharide.

Diploids injected with 0.9% NaCl

Fish/Day	0	1	2	3	5	11	15	19
4401	4.2	5.9	3.5	2.5	1.6	2.0	9.9	8.5
4402	8.0	6.3	3.7	2.4	5.4	12.8	9.1	6.4
4403	6.8		6.3	4.0	1.2	2.3	2.5	
4404	11.0	14.1	12.1	2.1	1.3	10.9		3.9
4405	4.7	3.1	4.1	1.9	3.3	6.7	11.6	9.7
4406	13.0	15.8	3.9		2.7	2.4	8.2	12.5
4407	8.9	6.1		1.4		9.3	11.8	10.4
4408	8.4	5.8	4.2	2.4	0.3		8.0	10.7
4409	6.0	6.9	5.7	2.7		3.8	5.1	6.6
4410	9.8	9.1	5.2	4.2	0.7	1.8	9.0	25.0
Mean	8.1	8.1	5.4	2.6	2.1	5.8	8.4	10.4

Triploids injected with 0.9% NaCl

Fish/Day	0	1	2	3	5	11	15	19
4421	5.5	5.9	3.3	5.4	1.6	7.7	14.3	14.0
4422	11.1	11.0	8.8	2.3	2.9		8.6	10.4
4423	9.3		6.9	4.8	3.1	4.3	7.5	
4424	6.6	7.3	4.6	3.7	1.7	3.2	7.3	11.1
4425	8.9	10.7	4.5	3.3	3.6		11.4	13.7
4576	12.0	4.4	5.4	2.9	1.6	4.4	9.1	5.3
4577	9.1	8.5	6.9	1.5	1.2	6.0	10.6	9.0
4578	16.9	15.2	15.3	10.0	3.2	5.9	18.3	23.8
4579	5.8	2.9	4.5	6.9	2.5	4.1	6.2	8.3
4580	8.5	9.8	4.7	3.1	4.4	6.8	8.4	8.4
Mean	9.4	8.4	6.5	4.4	2.6	5.3	10.2	11.5

TRIPLOIDS

DIPLOIDS

Diploids injected with lipopolysaccharide

Fish/Day	0	1	2	3	5	11	15	19
4411	7.4	3.2	0.3	2.1	4.3	5.8	9.0	8.8
4412	5.9	5.1		1.6	1.6	3.1		
4413	7.0	7.3	2.6	1.2	4.0	6.5	9.9	
4414	13.1	4.6	0.2	0.5	2.2	3.4	12.2	20.4
4415	6.7	7.4	2.5	1.1	3.2	5.1		11.8
4416	6.1	2.8	1.0	4.0	5.2	7.0	11.7	11.9
4417	7.1	3.8	1.9	2.0	3.7	7.9	9.8	12.6
4418	7.8	6.2	0.8	1.9	4.0	4.4		26.5
4419	9.7	5.5	2.0	1.8	5.2	9.0	15.0	15.4
4420	7.5	2.8	1.7	0.5	3.5	6.2	13.7	13.2
Mean	7.8	4.9	1.4	1.7	3.7	5.8	11.9	14.6

Triploids injected with lipopolysaccharide

Fish/Day	0	1	2	3	5	11	15	19
4581	5.1	3.2	2.1	2.0	3.7	5.4	7.6	3.6
4582	7.0	9.1	4.1	3.2	1.9	5.1	7.4	
4583	7.9	9.3	3.7	0.5	1.9	2.9	4.9	8.5
4584	12.9	9.4	2.0	0.3	2.7	3.3	8.0	5.7
4585	8.7	13.5	8.7	1.5	1.9	5.2	15.0	11.8
4586	8.9		3.6	1.1	2.5	4.3	4.4	6.1
4587	5.5	6.6	5.6	1.5	1.8	10.1	12.9	
4588	13.3	5.2	4.1	1.5	1.4	2.7		10.4
4589	10.4	4.0	3.7	2.1	2.8	6.7	10.5	
4590	5.9	6.1	3.0	0.8	1.5	2.6	4.7	6.5
Mean	8.6	7.4	4.1	1.4	2.2	4.8	8.4	7.5

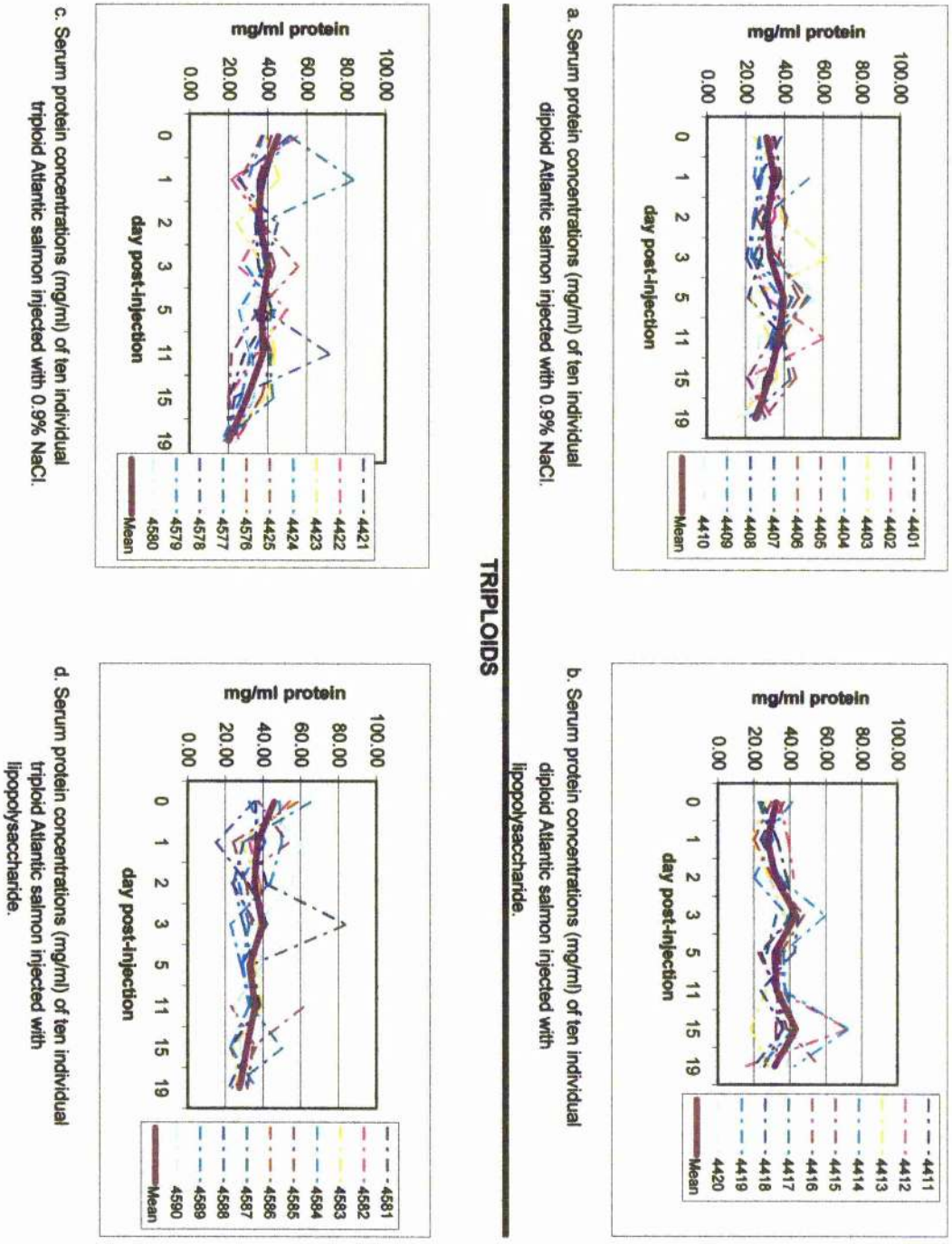
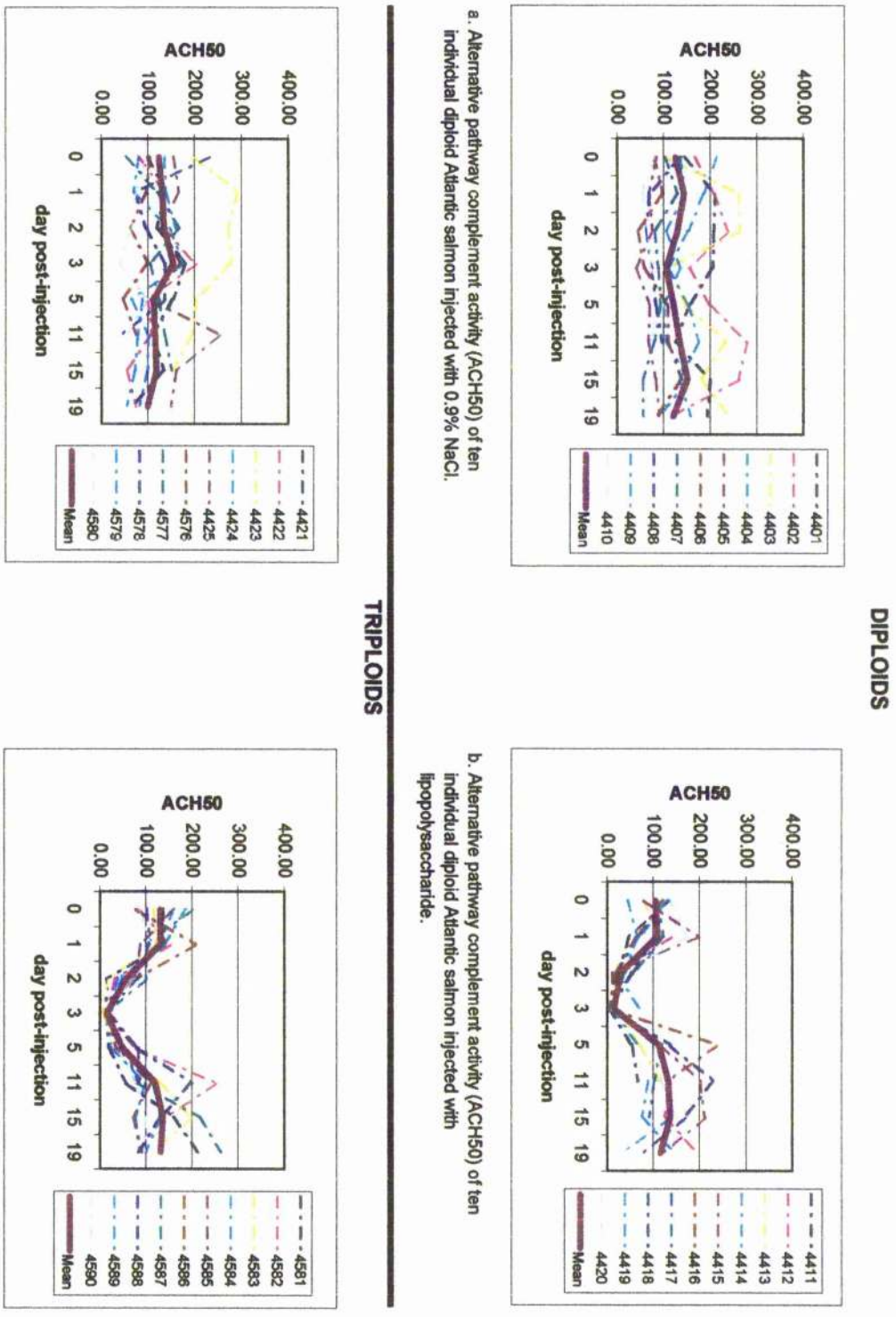


Figure 4.1 Serum protein concentrations, as monitored over 20 days, of individual diploid and triploid Atlantic salmon injected with either 0.9% NaCl or lipopolysaccharide.



a. Alternative pathway complement activity (ACH50) of ten individual diploid Atlantic salmon injected with 0.9% NaCl.

b. Alternative pathway complement activity (ACH50) of ten individual diploid Atlantic salmon injected with lipopolysaccharide.

c. Alternative pathway complement activity (ACH50) of ten individual triploid Atlantic salmon injected with 0.9% NaCl.

d. Alternative pathway complement activity (ACH50) of ten individual triploid Atlantic salmon injected with lipopolysaccharide.

Figure 4.2 Alternative pathway complement activity (ACH50 units/ml), as monitored over 20 days, of individual diploid and triploid Atlantic salmon injected with either 0.9% NaCl or lipopolysaccharide.

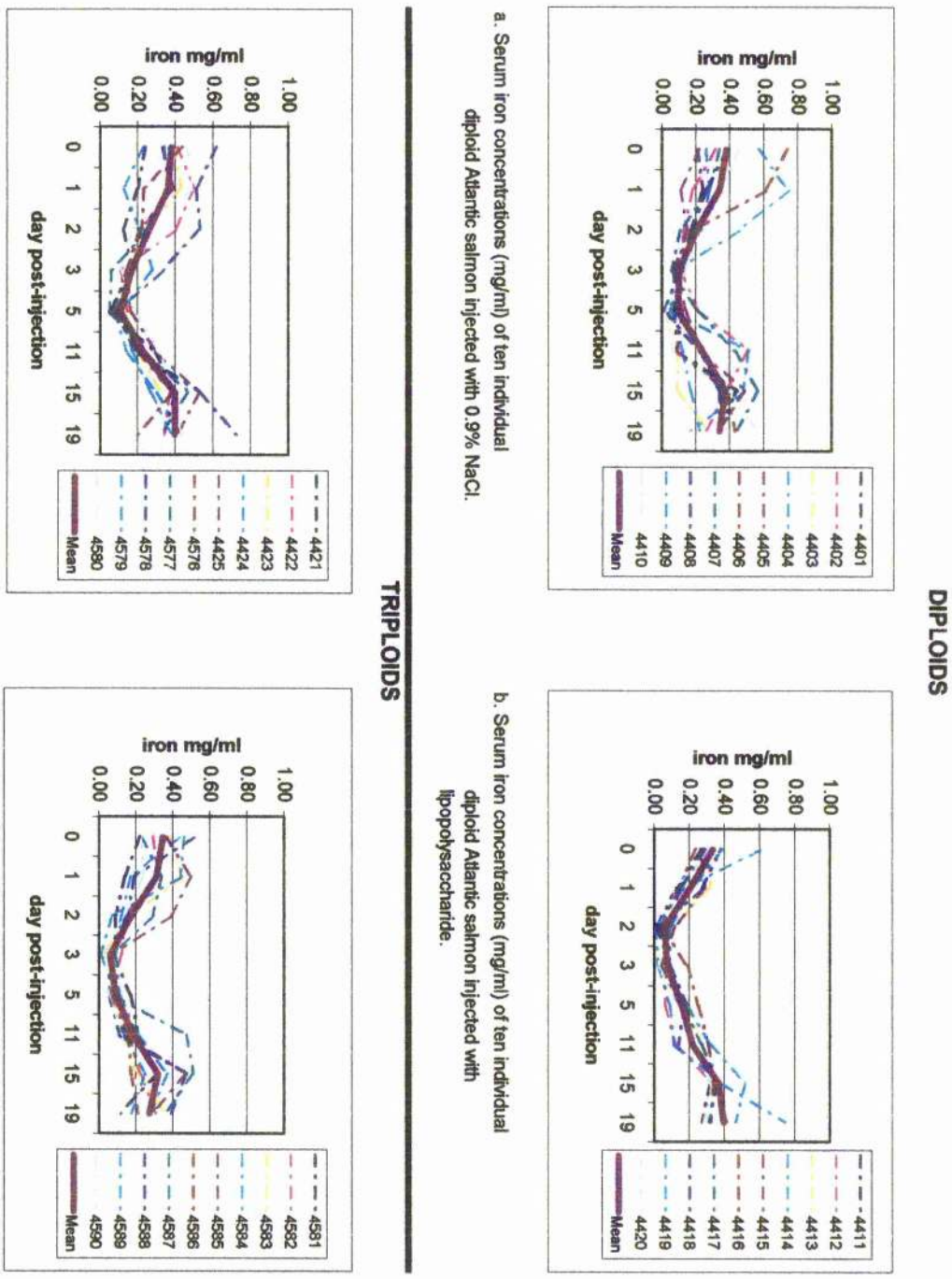


Figure 4.3 Total serum iron content (mg/ml), as monitored over 20 days, of individual diploid and triploid Atlantic salmon injected with either 0.9% NaCl or lipopolysaccharide.

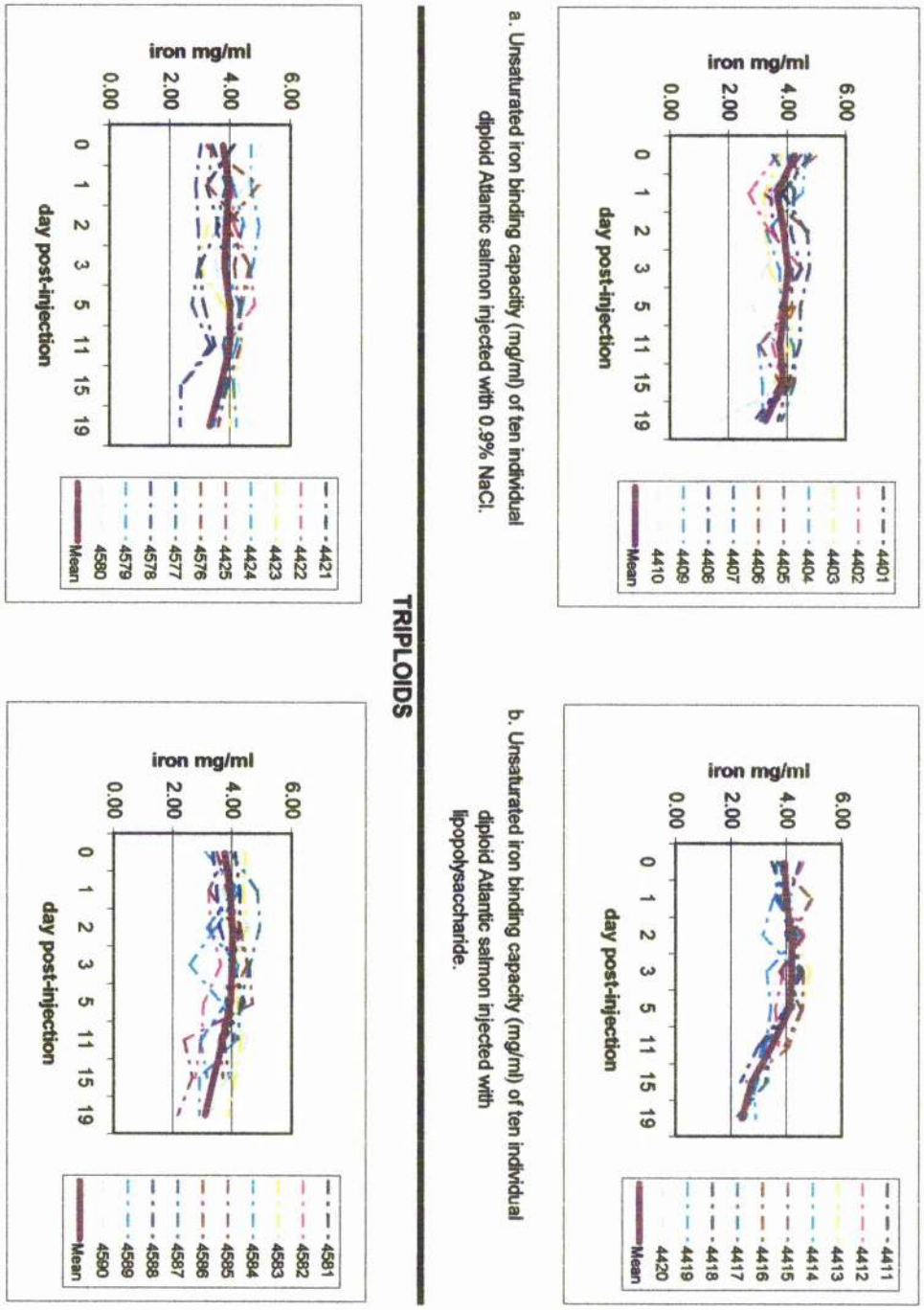


Figure 4.4 Unsaturated iron binding capacity (mg/ml), as monitored over 20 days, of individual diploid and triploid Atlantic salmon injected with either 0.9% NaCl or lipopolysaccharide.

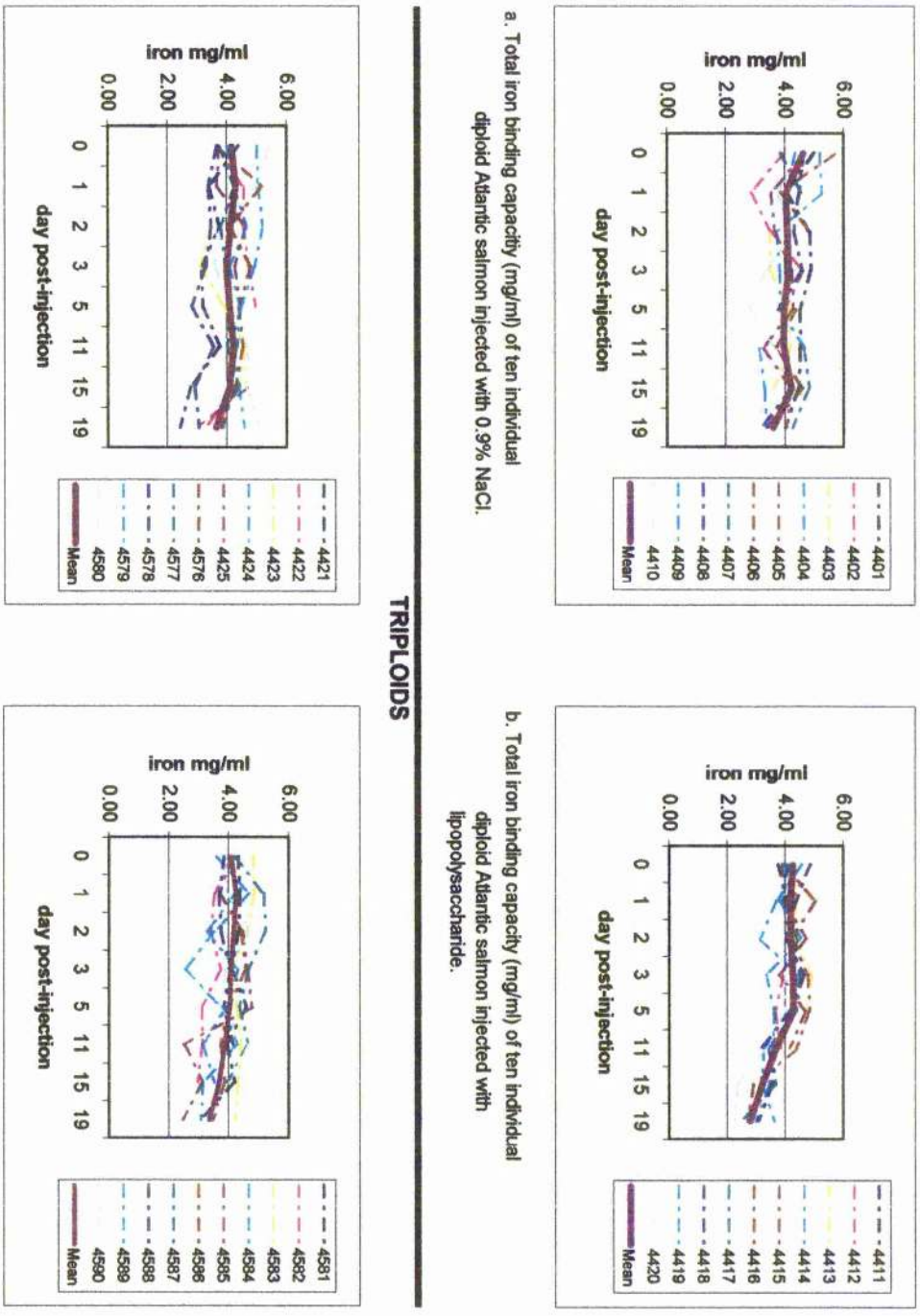


Figure 4.5 Total iron binding capacity (mg/ml), as monitored over 20 days, of individual diploid and triploid Atlantic salmon injected with either 0.9% NaCl or lipopolysaccharide.

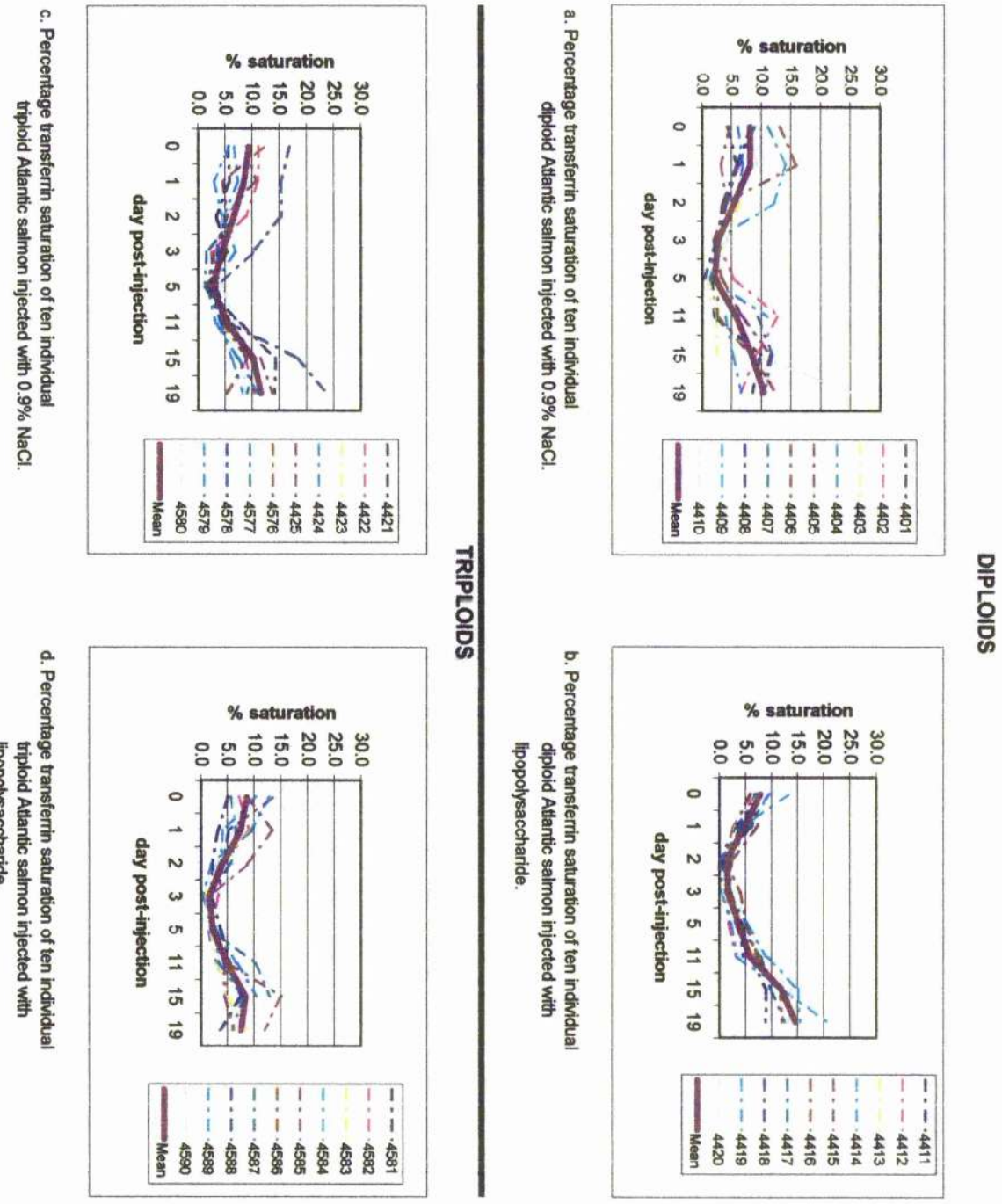


Figure 4.6 Percentage transferrin saturation, as monitored over 20 days, of individual diploid and triploid Atlantic salmon injected with either 0.9% NaCl or lipopolysaccharide.

4.3.6 Percentage transferrin saturation

Figure 4.6 shows the percentage saturation (%) for each individual in each treatment group over the course of the experiment.

This parameter was not analysed statistically since it is dependent on TI and TIBC. It is influenced mainly by TI and as such the graphs show similar patterns for percentage saturation to those seen for TI. For all treatment groups there is a decrease in percentage saturation which reaches a minimum at Days 2 and 3, before returning to baseline levels.

4.4 Discussion

Since there are no changes at Days 2 and 3 in serum protein concentration, it can be concluded that oedema has not occurred in response to the injection of LPS or saline. This is an important point to note. If oedema had occurred there would have been a corresponding decrease in serum protein concentrations, which in itself would equate to decreases in other humoral parameters per ml of serum. Changes which have therefore occurred over time in some or all of the treatment groups are therefore 'real' changes and not artifacts of oedema.

Changes in some of the non-specific, humoral immune parameters may have been caused by the intensive bleeding of the individual fish. Intensive bleeding over a short time period is stressful and stress effects may be involved. Also, bleeding removes the factors being measured from the fish and these factors may not be generated as quickly as they are removed. This may explain the slight decreases in serum protein and UIBC towards the end of the experiment.

Congleton and Wagner (1991) described a hypoferraemic response in rainbow trout. They found that iron concentrations and percentage saturation in plasma decreased after fish were injected with LPS but TIBC remained unchanged. This pattern of results was also found in the present study. TI and percentage saturation decreased in diploids and triploids but there were no changes in either UIBC or TIBC.

However, these changes occurred in the groups treated with LPS and the groups treated with saline. Congleton and Wagner (1991) found that fish injected with saline did not show any decrease in serum iron content. The present data may have resulted from an acute phase/hypoferraemic response being triggered by tissue damage caused by repetitive bleeding rather than the LPS, as discussed below. Congleton and Wagner (1991) used a method of bleeding fish which differs from that used in the present study. They cannulated the experimental fish and withdrew blood without the need for repetitive handling and anaesthetising of the fish. In contrast, fish in this study were regularly caught, anaesthetised and bled from the caudal vein. This difference may explain why both the saline and LPS treated groups showed a hypoferraemic response whereas Congleton and Wagner (1991) only found a hypoferraemic response in the LPS treated fish.

Tissue damage caused by bleeding may have induced the hypoferraemic acute phase response. LPS injection exacerbated this response causing it to persist for longer in the LPS treated groups. Some form of adaptation to the sampling procedure evidently occurred since fish in all groups recovered to normal. Serum dilution as a result of sampling cannot have caused the hypoferraemia since no change in total serum protein or complement levels was observed in the control groups.

TI, UIBC and percentage saturation did not differ between diploids and triploids at Day 0. However, the levels of TI and UIBC were lower than those reported in Chapter 3 and those reported by Ravndal *et al.* (1994). As discussed in Chapter 3, iron with-holding parameters may vary with diet and/or physiological status which may explain these differences. However, as investigated in Chapter 6, the differences may be attributable to family variation.

Fish complement has been shown to be activated *in vitro* by various agents in the absence of antibody. Such agents include lipopolysaccharide (LPS) which is derived from Gram-negative bacteria. This study shows that the alternative pathway (an antibody-independent pathway) of complement is also activated *in vivo* in response to LPS. The initial reaction occurred as quickly in triploids as it did in diploids, but

the former took slightly longer for levels to return to normal. The ability of the fish to respond to an additional insult during the recovery phase may be reduced. Therefore if the recovery phase is extend for the triploids, they would be more vulnerable for longer.

Although triploids take slightly longer to return to 'normal' ACH50 levels, there did not appear to be a delayed recovery of TI levels or percentage saturation in triploids. Instead there is evidence that TI levels of diploids decrease more quickly but recover at the same rate as triploids (ie. the hypoferraemic response is more rapid and more prolonged in diploids).

This may cause triploids to be more susceptible to bacterial infection and is consistent with the commercial experience, namely that triploids suffer increased losses particularly when faced with multiple insults. Differential response and recovery rates may be due to increased cell volume causing the process to be rate limited (eg. transfer distances are increased) or to differences in cell number.

- For ACP, the LPS caused a marked depletion in complement activity by Day 2 in both diploids and triploids, but recovery was slower in triploids. This maybe due to triploids having fewer cells which can produce the various complement proteins or because it takes triploid cells longer to release newly generated complement proteins due to increased intra-cellular distances.
- For TI, iron is possibly removed from serum by a lactoferrin-like protein. The delay in removal of iron from the serum in triploids is possibly due to increased intra- and inter-cellular transfer distances.

In an acute inflammatory response to either natural or experimental stimuli (eg. LPS), there is an accumulation of neutrophils and macrophages in the affected area. This response also occurs in response to tissue damage (Suzuki and Iida, 1992). Neutrophil lysis probably releases a range of anti-microbial factors into the area. However, cells also release factors without lysing. Neutrophils have been shown to degranulate (Lamas and Ellis, 1994). Although this has been observed to be a mostly

intracellular activity (degranulation into phagosomes) in fish, in mammals neutrophils release specific granules into areas of inflammation (Bullen and Armstrong, 1979). Mammalian neutrophils release lactoferrin which plays a major role in the inflammatory response (Otto *et al.*, 1992). As described in Chapter 3.1 and above, lactoferrin plays a major role in the hypoferraemic response of mammals and affects other immune mechanisms.

Although, this experiment shows that fish exhibit a hypoferraemic response, the mechanism of this response is unknown. The existence of a lactoferrin-like protein in fish may suggest that the mechanism of this response is similar to that of mammals. Chapter 5 explores the possibility of fish possessing such a protein.

5: Iron binding capacity of peripheral blood leucocyte lysates from diploid Atlantic salmon *(Salmo salar)* L.

5.1 Introduction

Iron with-holding is one of a range of non-specific defence mechanisms present in most vertebrates. It attempts to deny invading organisms and neoplastic cells access to iron which is essential for growth. One component of this iron-with-holding system in higher vertebrates is a group of iron binding glycoproteins collectively known as transferrins. This group includes serum transferrin and lactoferrin (Bezkorovainy, 1987).

Transferrin is principally found in serum or plasma, whereas lactoferrin is present in exocrine secretions of mammals (milk, tears, saliva, nasal exudate, bile etc.) and is a major component of the granules found in polymorphonuclear neutrophilic leucocytes (Bullen and Armstrong, 1979; Otto *et al.*, 1992; Weinberg, 1984). During infection, mobilisation of neutrophils to the sites of invasion occurs. Lactoferrin is released upon degranulation of these leucocytes, and after binding with iron in the infected region, the iron-saturated protein is ingested by macrophages. These then remove the iron and store it as ferritin (Weinberg, 1984; Weinberg, 1992).

Transferrin has been identified in fish including Atlantic salmon (Kvingedal *et al.*, 1993; Roed *et al.*, 1995) and the iron binding properties of fish serum and plasma have also been demonstrated (Congleton and Wagner, 1991; Hershberger and Pratschner, 1981; Ravndal *et al.*, 1994). Lactoferrin has not been identified in fish and the iron with-holding nature of fish neutrophils has not been previously investigated.

There are mechanisms present in fish which may suggest the presence of lactoferrin or lactoferrin-like activity. Congleton and Wagner (1991) have shown that fish exhibit an acute phase hypoferraemic response and this has also been shown in Chapter 4. The mechanism by which the hypoferraemic response is initiated in fish is unknown but in mammals it is mediated by interleukin-1 (IL-1). During an acute

phase response elevated IL-1 levels occur and stimulate the release of lactoferrin by neutrophils (Klempner *et al.*, 1978). The lactoferrin removes iron from serum transferrin forming lactoferrin-iron complexes, which are rapidly cleared from circulation. If a hypoferraemic response occurs in fish, it is likely that lactoferrin or a lactoferrin-like protein may exist as part of this response. Oral administration of bovine lactoferrin was found to increase chemiluminescence, phagocytic activity and respiratory burst activity of kidney phagocytes in rainbow trout (Sakai *et al.*, 1995) and increase resistance to bacterial and parasitic infection (Kakuta and Kurokura, 1995; Sakai *et al.*, 1993). Bovine lactoferrin also enhances chemiluminescence, phagocytic activity and respiratory burst activity, *in vitro* (Sakai, *et al.*, 1995). Lactoferrin in mammals has been shown to affect various immune functions including the production of cytokines, natural killer cell cytotoxicity, complement activation and lymphocyte proliferation (Brock, 1995) and may also aid bacterial killing by assisting iron to catalyse the formation of hydroxyl radicals (Weinberg, 1984).

This chapter describes a study into the iron binding potential of peripheral blood leucocytes in Atlantic salmon, *Salmo salar* L. One method of distinguishing lactoferrin and transferrin in mammals is studying the pH-dependency of removing iron from the iron-binding protein. Lactoferrin is relatively pH resistant and will not release bound iron when exposed to a lowered pH. In contrast, transferrin can be prompted to release bound iron by the addition of acid.

5.2 Methods

5.2.1 Peripheral blood leucocyte (PBL) lysate

Adult, diploid Atlantic salmon (mean weight 1.2 kg) were anaesthetised with tricaine methanesulfonate at a final concentration of 2mg l^{-1} (MS-222, Sigma) and blood collected from the caudal vein using an ice cold heparinised vacuette and 23g needle. The blood was kept on ice and diluted 1 part in 4 with L15 (Gibco) supplemented with 2% foetal calf serum (FCS,Sigma), 0.1% heparin (Sigma) and 100mg penicillin ml^{-1}/100IU streptomycin ml^{-1} (Pen-Strep, Gibco).

The diluted blood was layered onto a Percoll gradient (51% Percoll, Sigma in L15 culture medium) and centrifuged at 400g for 30 min at 4°C. Leucocytes were harvested from the Percoll interface and washed in L15 supplemented with 0.1% FCS, 0.1% heparin and 100mg penicillin ml^{-1}/100IU streptomycin ml^{-1} (L15 diluent). The washed cells were resuspended to the same volume in L15 diluent. Cell viability was estimated by the method of Absher (Absher, 1973). The cell suspensions were then adjusted to give a concentration of 2.5×10^7 cells/ml. These were centrifuged and the L15 diluent removed. The cell pellet was resuspended to the same volume in double distilled water and the cells allowed to lyse for 5 min at room temperature. The lysate was recentrifuged to remove cell debris and stored at -22°C until required.

5.2.2 Serum collection

Adult Atlantic salmon were anaesthetised with MS-222 and blood collected from the caudal vein using a serum sep vacuette (Greiner) and 23g needle. Blood was allowed to clot for at least one hour at room temperature and centrifuged at 1431g for 20 min to separate blood cells from the serum. It was then aliquoted and stored at -22°C until required.

5.2.3 Human control

A control human serum (Precipath U, Boehringer Mannheim) with a known total iron content (TI) and total iron binding capacity (TIBC) was used as control. The lyophilised serum was reconstituted in double distilled water and allowed to stand for 30 min before use. The TI is stated as averaging 1.28mg/ml within a range of 1.05-1.52mg/ml, and the TIBC 2.93mg/ml within a range of 2.40-3.46mg/ml.

5.2.4 Measuring total iron content and iron binding capacity - **Method 1**

Iron binding capacity and total iron content were determined using commercially available reagents (Boehringer Mannheim, Kit Nos. 125 806 and 759 422 respectively). A 1μg/ml iron standard solution was used as a positive control. This method measured iron binding capacity by the method described by Ramsay (1958)

and relies on measuring iron levels of a supernatant following the pH-dependent release of iron from the iron binding protein.

Total iron content assay

This assay measured iron concentration. An ascorbic acid/ detergent mix released the iron which was complexed with the iron binding protein and reduced it from Fe^{3+} into Fe^{2+}. The released iron then reacted with ferrozine to form a colour complex which could be measured spectrophotometrically.

Two reagents were used in this assay; the first was the 'reagent for blank' which was an ascorbic acid detergent solution and the second was the 'colour reagent' containing 0.016μM ferrozine.

For the sample (S), 1ml of colour reagent was added to 0.2ml of test solution (either serum, PBL lysate or supernatant from the TIBC assay detailed below) in a cuvette. For each sample, a sample blank (SB) was also made replacing the colour reagent with reagent for blank. For each set of experiments reagent blanks (RB) were prepared by adding 0.2ml of distilled water to 1ml of colour reagent. These were incubated for 10 min at room temperature and the absorbance then read at 560nm on an Ultraspec III spectrophotometer (Pharmacia).

To calculate ΔA (change in absorbance), firstly the absorbance of the sample blank was subtracted from the sample absorbance and then from this result the absorbance of the reagent blank was subtracted.

ie. $\Delta A = (A_S - A_{SB}) - A_{RB}$

For serum or PBL lysate, to calculate the concentration of iron (serum iron mg/ml) ΔA was multiplied by the factor 12.2, according to manufacturers instructions. For the supernatants from the TIBC assay, to calculate total iron binding capacity (TIBCmg/ml) ΔA was multiplied by the factor 36.6. The latent or unsaturated iron binding capacity (UIBC) was the TIBC less the serum iron concentration. Percentage saturation was estimated as serum iron divided by TIBC multiplied by 100.

Total iron binding capacity (TIBC) assay

Serum was treated with an excess of Fe^{3+} ions to saturate the iron binding protein, by addition of a 5µg/ml iron solution. This was incubated at 22°C for 30 min and uncomplexed iron was precipitated with magnesium carbonate, then incubated for 60 min at 22°C mixing approximately every 10 min. The mixture was then centrifuged at 804g for 10 min to remove the magnesium carbonate and the precipitated iron. The supernatant was removed, placed into an Eppendorf and centrifuged again to ensure that all precipitate was removed. The iron content of the supernatant was determined by the total iron content assay detailed above.

5.2.5 Measuring total iron content and iron binding capacity - **Method 2**

Total iron content and unsaturated iron binding capacity were determined using commercially available reagents (Sigma No. 565). The total iron content assay is similar to the assay detailed above, but has been miniaturised in order to make it time and sample efficient. UIBC was determined by a method adapted from Persijn *et al.* (Persijn *et al.*, 1971) and has also been miniaturised. This method saturates the iron binding protein with a known quantity of iron and measures the decrease in the free iron levels. This differs from method 1 in that it does not require the pH-prompted release of iron from the iron binding protein in order to measure the amount of bound iron.

Total iron content assay

Fifty microlitres of test sample (PBL lysate, serum or human control), standard (iron standard solution 5µg/ml) or blank (distilled water) were added to 250µl of buffer (acetate buffer pH4.5, with 1.5% hydroxylamine hydrochloride) in a flat well microtitre plate (Greiner). The microplate was shaken and the absorbance read spectrophotometrically with a Dias microplate reader (Dynatech) at 560nm to give the initial readings. Five microlitres of iron colour reagent (0.85% w/v ferrozine in hydroxylamine hydrochloride) was then added to every well and the plate incubated at room temperature with continuous, gentle shaking. After 10 min the microplate

was read again at 560nm to give the final readings. The total iron content was calculated as follows:

change in absorbance of test ΔA_{test} = final A_{test} - initial A_{test}

change in absorbance of standard ΔA_{std} = final A_{std} - initial A_{std}

TI (mg/ml) = $(\Delta A_{test} / \Delta A_{std}) X 5$

where 5 is the concentration of the iron standard(mg/ml)

Unsaturated iron binding capacity (UIBC) assay

Fifty microlitres of test sample (PBL lysate, serum or human control) was added to 200µl UIBC buffer (0.5M Tris buffer, pH 8.1 with added surfactant, Sigma) in a flat well microtitre plate. Fifty microlitres of iron standard was then added to all the test samples. Blanks consisted of 100µl of distilled water with 200µl of UIBC buffer and standards were 200µl of UIBC buffer with 50µl iron standard and 50µl distilled water. The microtitre plate was then shaken and the absorbance read at 560nm to give the initial readings. Five microlitres of iron colour reagent was then added to every well and the plate incubated at room temperature with continuous, gentle shaking to ensure thorough mixing in all wells. After 10 min the microplate was read again at 560nm to give the final readings. The UIBC was calculated as follows:

change in absorbance of test ΔA_{test} = final A_{test} - initial A_{test}

change in absorbance of standard ΔA_{std} = final A_{std} - initial A_{std}

UIBC (mg/ml) = $500 - (\Delta A_{test} / \Delta A_{std}) X 5$

where 5 is the concentration of the iron standard (mg/ml)

TIBC (mg/ml) = TI + UIBC

Percentage saturation (%) = (TI/TIBC) X 100

5.3 Results and Discussion

The total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC), total iron content (TI) and percentage saturation were assayed by two different methods, for PBL lysate, serum and the human control serum. Each sample was replicated at least twice but four times where possible. The results are presented in Table 5.1.

Using method 1 it was possible to determine the TI and TIBC of Atlantic salmon serum and human control serum, and subsequently the UIBC and percentage saturation. However, it was not possible to determine either TI or TIBC of PBL lysate.

Using the second method adapted from Persijn *et al.*, (1971), it was again possible to determine TI, TIBC, UIBC and percentage saturation of Atlantic salmon serum and human control serum. However, the UIBC (and TIBC) of salmon serum differed between the 2 methods with method 2 giving higher binding capacities. This differences is difficult to reconcile but may be due to thhe difference in how iron-binding capacity is measured. Method 2 is possibly the more accurate method as it monitors the amount of iron that is bound from an added iron source. With Method 1, however, the quantity of bound is measured after precipitation of unbound iron then the prompting of the release of iron by the lowering of pH. Therefore inaccuracies with method 1 could occur in 2 ways

- Not all iron is released by from the protein. However, this seems unlikely as TI does not differ between the 2 methods and this also requires the release of iron from the iron binding protein upon exposure to low pH.

- The precipitation of unbound iron by magnesium carbonate also causes the precipitation of 'loosely' bound iron. In mammals, the stability of the binding of iron molecules varies depending on the amount of iron bound to an iron-binding

molecule, and whether the iron is bound to the N or C terminus (Bezkorovainy, 1987). It is unknown whether this would also be true for fish transferrin.

Using method 2 it was also possible to determine the UIBC of PBL lysate but not TI. This assay to measure TI, and the assays for TI and TIBC in method 1, rely upon the dissociation of iron from the iron binding protein by addition of an acid buffer.

These results indicate the presence of an iron binding substance within the PBL lysate that does not release its iron at acid pH. This shows that this factor is lactoferrin-like. It is well reported that lactoferrin in mammals is resistant to acid and proteolytic degradation and can continue to bind iron at low pH (Weinberg, 1984).

Table 5.1. Total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC), total iron content (TI) and percentage saturation (%) of PBL lysate, serum and human serum control determined by two different methods. The mean and standard deviation are shown.

	PBL Lysate mg/ml n=5 fish		**Serum** mg/ml n=5 fish		**Human Control** mg/ml n=2 batches	
Method 1	**Mean**	**Std Dev.**	**Mean**	**Std Dev.**	**Mean**	**Std Dev**
TIBC	-	-	4.46	1.98	2.61	0.09
UIBC	-	-	3.22	1.96	1.26	0.10
TI	-	-	1.24	0.25	1.34	0.05
% sat	-	-	32.07	13.68	51.59	2.71
Method 2						
TIBC	1.17	0.26	7.84	1.48	2.67	0.36
UIBC	-	-	6.01	0.92	1.54	0.37
TI	-	-	1.82	0.84	1.13	0.21
% sat	-	-	22.60	7.66	42.65	9.12

6: Non-specific cellular immune responses of diploid and triploid Atlantic salmon

6.1 Introduction

6.1.1 Introduction

As described in Chapter 1, the non-specific immune system has cellular as well as humoral components. One of the fundamental differences between diploids and triploids is the change in cell size and Chapter 2 has shown that peripheral blood leucocytes in triploid Atlantic salmon are larger and fewer in number than diploid leucocytes. This Chapter presents an investigation which aimed to compare non-specific, cellular responses of diploid and triploid kidney macrophages.

6.1.2 Phagocytes

A variety of leucocyte types are involved in non-specific cellular defences of fish. These include monocytes/macrophages, granulocytes, non-specific cytotoxic cells and various accessory cells (Secombes, 1996). Macrophages and granulocytes are mobile phagocytes found in the blood and secondary lymphoid organs (kidney, spleen, gut-associated lymphoid tissue). Both are important in inflammation. However, it must be remembered that these leucocytes also have important roles in the specific immune response and their activity is not restricted to that described below. It must also be noted that although neutrophils and macrophages are considered to be 'professional' phagocytes, there are other cells that have phagocytic ability.

Inflammation is a response to microbial infection and/or tissue injury and includes the accumulation of leucocytes and fluid. Less mobile tissue granulocytes known as eosinophilic granular cells (EGC's) are also involved in the response to infection or injury at mucosal sites (Reite, 1998) (eg. gills, gut). There is an accumulation of neutrophils and macrophages in an inflamed area. The response is usually biphasic with an increase in blood neutrophils (neutrophilia) and their extravasation preceeding the appearance of monocytes and macrophages (Afonso *et al.*, 1998). These phagocytes form one of the major defence mechanisms against infection in

fish. Phagocytes engulf pathogens and then kill or deactivate them. There are three phases to the activity;

1. Attachment of the pathogen/particle to the phagocyte. Phagocytes have the ability to distinguish between targets. It can proceed in the absence of host factors but phagocytic activity is increased after opsonization of the pathogen/particles by complement in normal serum. This is because attachment is mediated by a variety of cell-surface receptors (Secombes, 1996).
2. Ingestion of the pathogen/particle. It can take two forms; engulfment or enfoldment (Secombes, 1996). The pathogen/particle is encased in pseudopodia and a phagosome formed which is drawn into the phagocyte.
3. Breakdown of the pathogen/particle. Following ingestion cytoplasmic granules and vesicles converge on and fuse with the phagosome. Their contents are discharged into the phagosome forming a phagolysosome and these have a variety of intra-cellular killing mechanisms (eg. Lysozyme activity, respiratory burst activity). However, it must be remembered that phagocytes also produce a range of killing mechanisms that function extra-cellularly (see below).

6.1.3 Phagocyte killing mechanisms

Reactive oxygen species

Stimulation of the phagocyte membrane, such as that which occurs during phagocytosis, may trigger the production of reactive oxygen species (ROS), which have potent anti-microbial activity (Chung and Secombes, 1988). Triggering may not be dependent on phagocytosis but instead on membrane stimulation since fish (Chung and Secombes, 1988), as with mammals, are able to produce ROS when they interact with both soluble (eg. Phorbol myriste acetate) and particulate stimuli.

Upon stimulation the respiratory burst occurs (an increase in oxygen uptake of the phagocyte). Part of the superoxide anion (O_2^-) generated is dismutated to hydrogen peroxide which is itself another potent anti-microbial agent especially as a source of hydroxyl free radicals (OH^-) (Chung and Secombes, 1988; Secombes, 1996). Other oxygen free radicals can also be released. All of these products are released by fish

phagocytes (Sharp and Secombes, 1993). As with phagocytosis, opsonisation, cytokines and eicosanoids can increase the production of ROS. Fish also produce macrophage activating factor (MAF), believed to be interferon gamma (IFγ) which can upregulate the macrophage respiratory burst and increase the killing activity of macrophages (Secombes, 1996).

Other killing mechanisms

In addition to the production of ROS, fish can also generate reactive nitrogen species (RNS) such as nitric oxide. Nitric oxide is produced via the action of nitric oxide synthase (NOS). NOS activity has been detected in fish (Secombes, 1996) and appears to be stimulated by exposure to live bacteria, LPS, Con A and phorbol ester.

Fish phagocytes also possess a range of enzymes that are anti-microbial, anti-viral or parasiticidal. These include lysozyme (Grinde, 1989) which is thought to be used in intra- and extra-cellular killing. Another important killing mechanism is the production of myeloperoxidase (MPO) by neutrophils. During phagocytosis neutrophils degranulate and release MPO which enhances the bactericidal capacity of macrophages (Lefkowitz *et al.*, 1997). MPO also binds with halide and H_2O_2 to form a cytotoxic triad (Lincoln *et al.*, 1995) that is toxic to bacteria and fungi. MPO may also have an important role as an immunoregulatory molecule and macrophages exposed to MPO show increased secretion of cytokines (Lincoln, *et al.*, 1995).

6.2 Materials and Methods

6.2.1 Media

The following media were used:

L15 diluent: L15 culture medium (Sigma) supplemented with 0.1% heparin, 2% foetal calf serum (FCS, Sigma), 100mg penicillin ml^{-1}, and 100IU streptomycin ml^{-1} (Pen-strep, Sigma).

L15 wash: L15 culture medium (Sigma) supplemented with 0.1% heparin, 0.1% foetal calf serum (FCS, Sigma), 100mg penicillin ml^{-1}, and 100IU streptomycin ml^{-1} (Pen-strep, Sigma).

Hanks balanced salt solution (HBSS, Sigma).

6.2.2 Sample Collection

Kidney macrophages

Head kidney macrophages were collected according to the method described by Secombes (1990). Fish were killed by anaesthetic overdose. The gills were cut and the fish allowed to bleed. The head kidney was removed under aseptic conditions and disaggregated into ice-cold L15 diluent. The kidney suspension was layered onto a 34/51% discontinuous Percoll density gradient and centrifuged at 400g for 30 min at 4°C with no brake applied. Cells were harvested from the 34% and 51% Percoll interface and washed twice in L15 wash. Viable cell numbers were estimated using the method of Absher (1973). Cell suspensions were adjusted to the required cell concentration in L15 wash and 100µl/well of the cell suspension plated into sterile tissue culture microtitre plates (Nunc). The lidded microtitre plates were incubated overnight at 15°C in a humid box. Non-adherent cells were removed by aspiration and the adherent monolayers washed with L15 wash. The monolayers were then ready for use.

6.2.3 Assays

Respiratory burst assays

Phagocytes possess a membrane enzyme, NADPH oxidase, which is capable of reducing molecular oxygen to superoxide anion (O_2^-) during the respiratory burst. The respiratory burst will only occur after membrane stimulation. Particulate stimuli can achieve this but in this case a soluble stimulant (Phorbol myristate acetate, PMA) was chosen for ease of repeatability. The specificity of the assays can be demonstrated by preventing reduction of the reagents with the addition of exogenous superoxide dismutase (SOD). Reduction is prevented due to the SOD dismutating any O_2^- generated to H_2O_2.

Nitroblue tetrazolium reduction

Intracellular O_2^- production of macrophage monolayers was detected by measuring the reduction of nitroblue tetrazolium (NBT, Sigma) according to the method of Secombes (1990). Monolayers were prepared by plating out 100µl of $1x10^7$ cells/ml as described above, allowing overnight adherence, removing non-adherent cells and

washing with phenol-red free HBSS. The HBSS was removed and the monolayers exposed to 100µl of either 1mg/ml NBT (dissolved in HBSS), NBT + PMA (1µg/ml), or NBT + PMA + SOD (300µg/ml) for various time intervals. The reaction was then stopped by the removal of the reagents and addition of 100% methanol. The monolayers were then washed 3 times with 70% methanol and the intracellular formazan resolubilised by the addition of 120µl dimethyl sulphoxide (DMSO) and 140µl 2M potassium hydroxide (KOH). The absorbance at 630nm was determined spectrophotometrically using DMSO/KOH alone as blank. Additional wells of cells were treated similarly but without exposure to the substrates and the monolyers lysed according to the method described by Secombes (1990). Cell nuclei were counted using a haemocytometer and numbers of cells per well estimated. Results were adjusted to 10^5 cells per well and expressed as OD/10^5 cells.

Phagocytosis assay

The phagocytic activity of kidney macrophages was evaluated using a microscopic counting technique. A monolayer of cells was prepared by plating 100µl/well of $1x10^7$ cells/ml into a 16-well chamber slide (Nunc), allowing overnight adherence, removing non-adherent cells and washing with HBSS. A suspension (100µl) of heat-killed yeast (*Saccharomyces cerevisae*, Sigma) at a concentration of 1 x 10^7 cells ml^{-1} in HBSS was added to each well and incubated in a humid box at 15°C for various time intervals. After the required time, the monolayer was washed 3 times with HBSS and the cells fixed with 70% methanol. The wells and gasket of the chamber slides were then removed and the slides stained using Hema Gurr (BDH). The slides were examined using x100 oil immersion objective on a light microscope. Counts were made of the number of cells which had phagocytosed in a total population of 100 cells as well as the number of yeast cells within each phagocytic cell. Results were expressed as percentage number of phagocytic cells engulfing one or more yeast particles and the mean number of yeast cells engulfed per phagocytic cell (phagocytic index).

6.2.4 Measurement of macrophages

Macrophage monolayers on glass slides were examined using x100 oil objective on a light microscope. Ten cells were measured (cell length and cell width) for each of 2 diploid and 2 triploid fish using a graticule.

6.3 Results

6.3.1 General

In all cases it was noted that the yield of cells (when harvested from the Percoll gradient) from a triploid kidney was lower than that of an equivalent diploid kidney.

6.3.2 Intracellular respiratory burst activity

Results are shown in Figure 6.1 (n=3 diploids; n=4 triploids). Results are expressed as the difference in mOD between PMA stimulated cells and cells exposed to PMA + SOD.

Activity increased to a maximum at 30 min post-addition of substrate for diploid and triploid macrophages. The mean activity of the triploid and diploid groups at 30 min was not statistically significantly different and there were no significant changes in activity after 30 min. The rate of activity did not differ between groups.

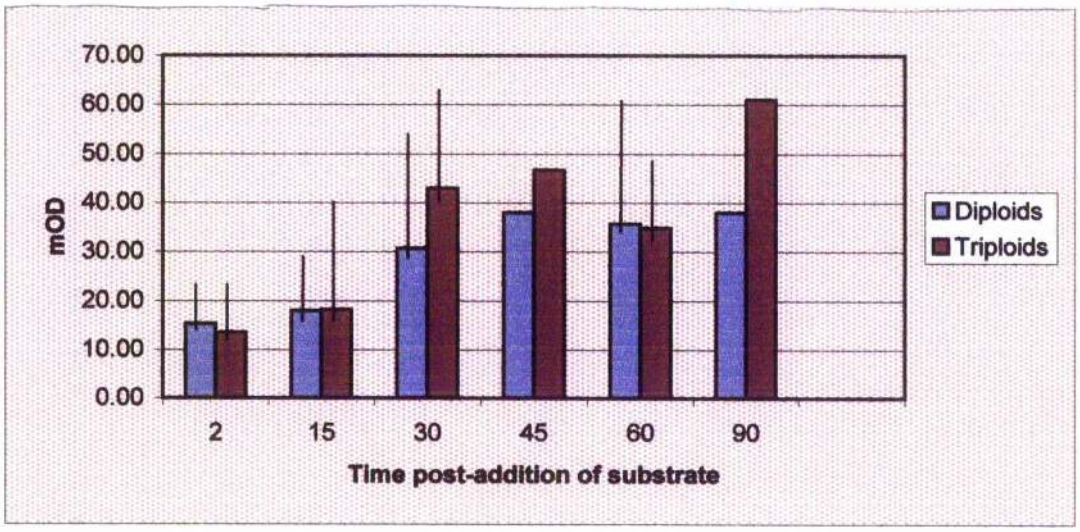


Figure 6.1 Intracellular respiratory burst activity (mOD 10^5 cells) of diploid and triploid Atlantic salmon kidney macrophages.

6.3.3 Phagocytosis Activity

Results are shown in Table 6.1 (n=10 diploids; n=10 triploids).

The percentage of the macrophage population which were phagocytic did not differ significantly between diploids and triploids (p=0.44). There was 61.82% (diploids) and 63.72% (triploids) of the populations which were phagocytically active.

However, there were statistically significant differences (p<0.001) in the phagocytic index (average number of yeast phagocytosed by active cells) between diploids and triploids. Triploids had a higher phagocytic index of 1.95 (+/- 0.2) whilst diploids had an index of 1.5 (+/- 0.2). The phagocytic index of the diploid macrophages is equivalent to two thirds of the phagocytic index of the triploid macrophages.

6.3.4 Size of macrophages

Results are shown in Table 6.2. Triploid macrophages are significantly larger than diploid macrophages (p=0.001). The diploid/triploid ratio of cell length is 0.77 and for cell width is 0.58. If the theoretical diploid/triploid ratio is 0.66 then the width is slightly smaller and length slightly larger than expected.

Table 6.1 Phagocytic activity (%) and index of diploid and triploid Atlantic salmon kidney macrophages.

Fish No.	Diploids		Triploids	
	Phagocytic Activity (%)	Phagocytic Index	Phagocytic Activity (%)	Phagocytic Index
1	62.00	1.51	53.84	2.10
2	57.54	1.19	57.69	2.21
3	65.62	1.72	77.77	1.67
4	63.46	1.71	53.84	1.99
5	63.11	1.24	68.57	2.02
6	67.64	1.56	78.49	1.84
7	61.61	1.73	53.95	1.81
8	59.01	1.43	70.58	2.28
9	56.71	1.72	67.69	1.73
10	53.08	1.61	54.76	1.90
Mean	**61.86**	**1.53**	**63.72**	**1.95**
Std Err	**1.21**	**0.07**	**3.17**	**0.06**

Table 6.2 Mean sizes of diploid and triploid Atlantic salmon kidney macrophages (μm).

	Mean cell length (μm)	Std Err cell length	Mean cell width (μm)	Std Err cell width
Diploid 1	6.0	0.63	5.5	0.45
Diploid 2	6.0	0.26	4.8	0.31
Triploid 1	8.4	0.58	9.7	0.89
Triploid 2	7.3	0.42	8.0	0.45

6.4 Discussion

6.4.1 Macrophage size and number

One of the major differences between diploid and triploid fish (including Atlantic salmon) is cellular size. For many cell types, triploid cells are one third larger than diploid cells due to the increase in genetic material and there are also a third fewer cells (Benfey, 1997; Small and Benfey, 1987). Peripheral blood leucocytes (PBL's) in triploid Atlantic salmon are also larger and fewer in number than diploid PBL's (Chapter 2). This study has also shown that kidney macrophages are larger in triploid Atlantic salmon than in diploids. This size increase is mainly as a result of increase in cell width. Observations also suggest that macrophages are fewer in number in triploids. However, if analogies are made in comparison with the data on peripheral blood leucocytes from Chapter 2, it is probable that the proportion of kidney cells which are macrophages does not change and that all cell types are fewer in number.

6.4.2 Phagocytosis

Triploids have previously been reported to have an increased phagocytic activity for an individual cell but this appeared to be balanced by the lower total number of cells (Yamamoto and Iida, 1995b). This has also been shown in this study. For a given number of cells, diploids and triploids have the same percentage which will phagocytose. But each phagocytic triploid cell can take up more yeast than a diploid cell. This difference between diploid and triploid cells is almost one third (1.26), suggesting that if the triploids possess one third less macrophages, then this is balanced by the ability to phagocytose one third extra per cell.

The volume of a triploid cell increases by a third (Small and Benfey, 1987). But the increase in surface area would not be a third and, in total, triploids would have a lower surface area to volume ratio than a diploid, once the number of cells has been accounted for. The phagocytic index (the amount taken up by a phagocytic cell) of triploid macrophages was higher than that of diploid macrophages in this experiment. The increase in phagocytic index is about one third. This suggests that the increase is related to the increase in cell volume rather than the increase in

surface area of triploid cells compared to diploid cells (ie. That the extra yeast are taken up as a result of increased cell volume rather than increased surface contact).

6.4.3 Respiratory Burst

The amount of intracellular O_2- produced per macrophage appeared to be similar for diploids and triploids. Kinetics of NBT reduction was also the same for diploids and triploids. Therefore differences in cell size did not apparently have an effect on induction of the respiratory burst.

Although the ability of a triploid macrophage to phagocytose is increased, it does not have a corresponding increase in intracellular respiratory burst activity. With no additional considerations, this would seem to put triploids at an overall disadvantage

The question then arises-while triploid macrophages can phagocytose more potential pathogens, are they comparatively less able to neutralise/kill these pathogens?

Superoxide anion and hydrogen peroxide (produced during the respiratory burst) are potent bactericidal agents (see Chapter 6.1). Respiratory burst activity is considered an important mode of microbial killing in mammalian macrophages and these factors produced during respiratory burst are also produced by fish macrophages (see Chapter 6.1). If the total macrophage population of triploids has an overall lower respiratory burst activity (fewer cells, each with the same respiratory burst activity as diploids), this could have fundamental consequences on the efficiency of the triploid immune system to combat pathogens. In the case of most pathogenic microorganisms, killing by fish phagocytes is an intracellular process (Secombes, 1996). Killing of some *Aeromonas salmonicida* strains was found to be optimal following internalisation, since the presence of cytochalasin B (an inhibitor of phagocytosis) reduced macrophage killing activity (although not completely) (Sharp and Secombes, 1992). This study by Sharp and Secombes (1992) led them to believe that intracellular respiratory burst activity was important for the killing of some strains *of A. salmonicida.* They also presented evidence that inhibition of the respiratory burst resulted in inhibition of killing of these strains.

There is a threshold of activation of the respiratory burst (corresponding to a minimum quantity of superoxide anion) required for the killing of some pathogens. Unactivated macrophages are only able to kill avirulent strains of *A. salmonicida* but once activated they are able to kill virulent strains (Sharp and Secombes, 1992). However, the amount of superoxide required to kill a pathogen varies between strains (Karczewski *et al.*, 1991). Considering all this information, if a triploid macrophage can ingest more pathogens but does not have an increased respiratory burst, it may be possible that it is unable to kill all those pathogens which have been phagocytosed, if this critical threshold is not reached.

Macrophages release extracellular superoxide as well as the intracellular superoxide determined in this study. It is possible that in contrast to an overall lowered intracellular burst in triploids, the extracellular burst is increased.

However, it may be that the triploid macrophages in this experiment were not stimulated enough to induce their maximal respiratory burst. In this experiment, a standard amount of soluble stimulant was exposed to the same number of cells. The concentration of PMA used may have been optimal to stimulate diploid macrophages into maximal respiratory burst but because there was the same number of triploid macrophages per well, there would have been a greater surface area of triploid macrophages. Therefore, the PMA concentration used may have been suboptimal for maximal stimulation of the respiratory burst in triploid macrophages. It is therefore important to test higher PMA concentrations on triploid macrophages.

Stimulation of a macrophage to make it burst is regulated by cytokines and eicosanoids and these factors may be affected by host derived factors (Secombes, 1996). In an immune response it may be possible that there would be more stimulation of the surface of the macrophages (since surface area per cell is increased) resulting in a higher respiratory burst in the triploid cells since they can phagocytose more (measuring the respiratory burst in the yeast phagocytosis experiment might, in retrospect, have cast light on this). Opsonisation can increase respiratory burst activity (Chung and Secombes, 1988). Additionally, macrophages

can be 'activated' which increases their killing activity. Activation is caused by macrophage activating factor (MAF) which is produced by surface Ig $^{-}$ lymphocytes (Secombes, 1996). There is also evidence that suggest other cytokines can interact with MAF and enhance respiratory burst activity (Secombes, 1996). It may be possible that during an immune response these additional factors within the cellular immune response may augment the respiratory burst of a triploid cell, thereby negating the overall disadvantage of a reduced respiratory burst.

7: Determination of antibody dynamics of diploid and triploid Atlantic salmon

7.1 Introduction

As mentioned earlier, it is important to consider the magnitude and speed of an immune response rather than the mere existence of a defence mechanism when considering the ability of an organism to successfully combat pathogenic invasion. Similar to Chapter 4, it was considered that, since one of the major differences between diploid and triploid fish is that triploids have larger and fewer cells, this may have effects on the kinetics of their immune response. Chapter 4 presented results from a study of the kinetics of the non-specific humoral immune response. This Chapter presents a study on the kinetics of the specific immune response by monitoring the dynamics of specific antibody production in response to a commercial vaccine.

7.1.1. Specific immune system

Specific (protective or adaptive) immunity is defined by its ability for memory and specificity. Similar to the non-specific immune system, it consists of cellular and humoral parameters which can work alone or in conjunction with each other.

Humoral immunity is mostly based on antibody production and is described below. Cell-mediated immunity is mediated by a different path to that of humoral immunity and is essentially based on T-lymphocytes rather than B lymphocytes (see below).

7.1.2. Antibodies of fish

Antibody responses in mammals result from a co-ordinated activity of B and T lymphocytes, accessory cells (eg. monocytes and macrophages), and cytokines (released mainly from CD4+ T helper (Th) cells) (Manning and Nakanishi, 1996). The hallmarks of mammalian antibody responses include rapid secretion of antigen-specific antibodies, isotype switching, affinity maturation (a function of somatic hypermutation and selection of high affinity B cell clones), as well as the development of efficient memory cells which mediate an improved secondary

response. Many of these features have been identified in fish, including functional antigen presentation by accessory cells (Vallejo *et al.*, 1992), involvement of the major histocompatibility (MHC) class II molecules, presence of a T cell receptor (TCR) (Partula *et al.*, 1994) and surface immunoglobulin (sIg) on B cells (Koumans Van Diepen *et al.*, 1994). However, isotype switching and significant affinity maturation do not occur in fish (Kaattari, 1984).

Fish above the level of agnathans produce antibodies. The structure, nomenclature and organisation of their Ig genes are well established (Pilstrom and Bengten, 1996; Warr, 1995). As a generalisation, fish tend to produce lower affinity and less diversity of antibodies than do birds and mammals. Similarly, the memory component of their antibody responses is much weaker than that of birds and mammals (Pilstrom and Bengten, 1996; Warr, 1995).

Ig is either secreted by B cells or integrated into the membrane of B cells to act as an antigen receptor (sIg) (Warr, 1995). Specific antibodies have been found in serum (Davidson *et al.*, 1993), plasma, bile and mucus of fish. In contrast to mammals, it is generally held that fish possess only IgM.

7.1.2 Influences

The production of antibody (as with all aspects of the immune system) is affected by a variety of factors. In the context of the immune response the terms influence, regulate and modulate refer to an alteration or adjustment of the nature or magnitude of the immune response. The alteration or adjustment may be positive or negative. The factors which can influence the immune response can be extrinsic or intrinsic factors.

Extrinsic factors

Extrinsic (or environmental) factors that influence antibody production include temperature, antigen dose and route of immunisation.

Low water temperatures have been reported to inhibit, reduce or retard lymphocyte responsiveness or antibody production (Cuchens and Clem, 1977; Manning and Nakanishi, 1996). Antigen processing and recognition are relatively temperature independent (Avtalion *et al.*, 1976). This is also true for proliferation and differentiation of B-cells. However, the cellular interactions between antigen processing and B-cell response may be temperature sensitive as are antibody synthesis and release. T-helper cells are those which are mostly affected by temperature, whilst B cells, T memory cells (Miller and Clem, 1984) and accessory cells (Vallejo, *et al.*, 1992) are unaffected by temperature. It is important to note however, that a study by Lillehaug *et al.* (1993) showed that although low temperatures reduced antibody production against *Vibrio salmonicida*, protection was actually enhanced. Other evidence suggests that non-specific cytotoxic cell activity can be enhanced at lower temperatures (Le Morvan-Rocher *et al.*, 1995) which may compensate for the inhibitory effects on specific responses.

Intrinsic

Intrinsic (or internal) factors that influence antibody production include age (stage of development) and genetics.

The age and size of the fish can influence the responsiveness to antigen stimulation. The developmental stage of the lymphoid organs is very important in determining responsiveness. By contrast, if a critical number of immune cells (macrophages, T or B lymphocytes) are required for responsiveness, then mass of the lymphoid tissue may be the critical criterion. The reports on minimum ages/sizes to facilitate protection or antibody production vary with species and the route of administration (Joosten *et al.*, 1995).

With regards to genetic background, the antibody responses to antigens in outbred fish are frequently reported to be highly variable, ranging from low or non-responsiveness to high levels. The variability of specific antibody responses of homozygous fish is reduced in comparison to heterozygous fish, and in addition the

antibody diversity (as measured by the banding patterns generated by iso-electric focusing) is reduced (Cossarini-Dunier *et al.*, 1986; Desvaux *et al.*, 1987).

7.2 Materials and Methods

7.2.1 Experimental design

The vaccination trial was executed at Matre Aquaculture Research Station, Havforskningsinstituet, Norway. All fish were from one family group, 'E9' (full-siblings), and were mixed sex pre-smolts weighing between 70 and 120g at the start of the trial. They were kept in 1.5 x 1.5m square tanks supplied with flow-through fresh water and fed on a commercially available pellet feed. Water temperatures are shown in Table 7.1.

Four groups of fish were treated as follows (outlined in Table 7.2): diploid injected with saline, triploid injected with saline (as controls); diploid vaccinated, triploid vaccinated. Vaccinated fish were injected with a commercial quadruple vaccine (Intervet Norbio) against cold water vibriosis, *Vibrio anguillarum*, *Aeromonas salmonicida*, and infectious pancreatic necrosis virus (IPNV) according to standard protocols. At week 6, vaccinated fish were boosted and non-vaccinated fish re-injected with saline. Twenty fish from each group were killed by a blow to the head and sampled for serum on the following occasions; before treatment (week 0), weeks 4, 8, 12, 16, 20, 24 post-injection.

7.2.2 Sample collection

Blood was collected from the caudal vein of fish anaesthetised with metamidate, using a needle and 1ml syringe. Blood was placed into a centrifuge tube and allowed to clot at room temperature. The clotted blood was centrifuged and serum collected. Serum was stored at -80°C and transported to Aberdeen on dry ice. In Aberdeen the serum was stored at -20°C until analysed. Sera were analysed for specific antibody titres to *Aeromonas salmonicida* by bacterial agglutination.

Table 7.1 Mean, minimum and maximum water temperatures (°C) recorded daily from experimental tanks, for the duration of the trial.

Week	Mean (°C)	Minimum (°C)	Maximum (°C)
0 – 4	6.6	6.2	7.0
4 – 8	6.3	5.8	7.0
8 – 12	6.3	5.6	6.5
12 - 16	6.5	6.4	6.6
16 - 20	7.1	6.8	7.1
20 - 24	9.1	8.3	11.9

Table 7.2 Vaccination regime for the four treatment groups.

Group Number	Ploidy	Treatment at week 0	Boost at week 6
A	Diploid	0.1ml 0.9%NaCl	0.1ml 0.9%NaCl
B	Triploid	0.1ml 0.9%NaCl	0.1ml 0.9%NaCl
C	Diploid	0.1ml Vaccine	0.1ml Vaccine
D	Triploid	0.1ml Vaccine	0.1ml Vaccine

7.2.3 Agglutination Assay

Preparation of bacterial suspension

A non-autoagglutinating strain of *Aeromonas salmonicida* subsp. *salmonicida* (MT 004) was grown from stock on tryptone soy agar (TSA, Sigma). Once established on agar, colonies were used to inoculate tryptone soy broths (TSB, Sigma). The broths were incubated at 22°C whilst shaking at 140rpm for 48 h. Formalin was added to 0.5% v/v and the broths again incubated at 22°C, 140rpm for 24 h. The bacteria were washed in saline (0.9% NaCl in distilled water), the concentration adjusted to 10^9 bacteria per ml in saline (optical density at A540nm is 1.0), centrifuged and the volume adjusted to give a concentration of 10^{10} bacteria per ml.

Assay

Sera were serially diluted (neat to 1:2048) in saline to a final volume of 25µl in V-form 96-well microtitre plates and 100µl of the bacterial suspension was added (total of 10^9 cells/well). The plates were incubated at 4°C overnight and the titres were scored as the first well in which the bacterial pellet in the V-well, flowed from the bottom of the V when the plate was tilted and viewed from below.

7.2.4 Statistical Analysis

Mean titres were examined by analysis of variance (ANOVA) on GENSTAT.

7.3 Results

Negative control serum produced $\log_2$ titres of between 2 and 4. Positive control serum produced $\log_2$titres of between 5 and 7.

The results of the test sera are shown in Figure 7.1. Mean $\log_2$titres are shown in Table 7.3. Because of the wide individual variation in the antibody titres, the graphs show the number of individual fish with a particular titre at each time point for each treatment group. This allows the interpretation of the data to be based on the study of the distribution of titres rather than the mean titre expressed by a group at each time point. However, mean titres have also been examined.

To aid with the interpretation of the data, limits have been imposed to determine negative and positive specific antibody responses. It must be noted that these limits have not been statistically calculated but instead empirically derived from the test and control results. No control fish had a $\log_2$titre above 5. Therefore, those titres of 1 to 3 are considered negative specific antibody responses, titres of 5 to 8 are considered positive specific antibody responses and titres of 4 maybe either negative or positive specific antibody responses.

For the unvaccinated control groups the antibody titres ranged from 1 to 5. Most are below 3. However, there is a trend of increasing antibody titre over time for both control groups, with this increase being higher in the triploids. The highest antibody titres for individuals in the unvaccinated groups occurred at week 16 ($\log_2$ titre = 5) for the triploids and week 20 ($\log_2$ titre = 4) for the diploids. There are no consistent differences between diploids and triploids. At week 0 there are no statistical differences ($p=0.29$). However at week 24 triploids have a statistically higher titre than diploids ($p=0.039$). There are no statistical differences for the intervening weeks.

For the vaccinated groups the antibody titres showed a clear increase over time. At week 4 the specific antibody responses were negative (below $\log_2 4$) for both diploids and triploids. At week 8 some positive responses were evident. Triploids have a statistically significant higher agglutinating response than diploids at this time point ($p=0.002$). At week 16, most fish showed positive antibody responses. The trend of increasing agglutinating titres continued with the highest titres occurring at week 24 ($\log_2$titre = 8) for some individual triploids and week 20 ($\log_2$titre = 8) for the diploids. Triploids have a higher agglutinating titre at week 24 ($p=0.039$) than diploids.

Table 7.3 Mean agglutinating log_2titres for diploid (D) and triploid (T), vaccinated (V) and unvaccinated (unV) Atlantic salmon over 24 weeks (n=20 for each group at each time point).

Week	DunV	TunV	DV	TV
0	1.56	1.78		
4	2.35	1.95	2.35	2.30
8	2.55	1.90	3.10	3.70
12	2.70	2.50	4.10	4.45
16	2.50	3.45	5.45	5.95
20	3.05	3.00	5.80	5.75
24	1.80	2.40	5.05	5.95

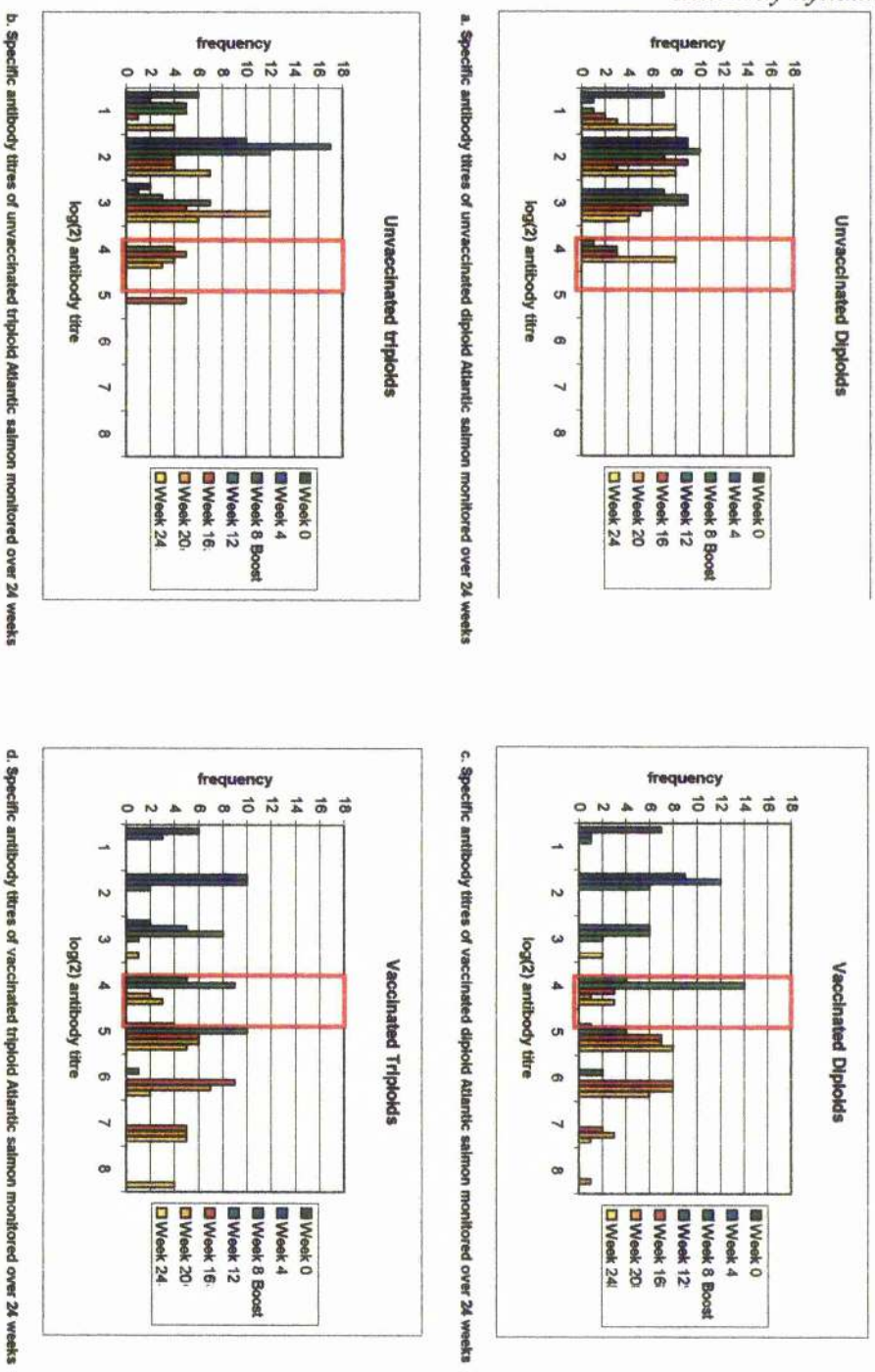


Figure 7.1 The dynamics of specific antibody production of vaccinated (commercial vaccine) and unvaccinated (0.9% NaCl) diploid and triploid Atlantic salmon. (Red Box: titres to the left of the box are considered negative responses; titres to the right are considered positive responses; those within the box maybe positive or negative).

7.4 Discussion

Specific antibodies are those which are specific to the antigen studied; in this case the vaccine. To monitor the specific response to the vaccine, agglutinating antibodies to whole bacterial cells were measured. Results may not have been the same if another bacterial component had been chosen instead.

The unvaccinated fish showed a slight increase in antibody levels over time with a maximum individual fish titre of $\log_2 5$. This increase in agglutination titre may reflect an increase in non-specific antibodies or lectins. Lectins are proteins which agglutinate and/or precipitate glycoconjugates (Yano, 1996). Antibody agglutination titres in vaccinated fish were therefore considered positive if the $\log_2$titre was 5 or more.

The vaccinated groups showed a clear increase in agglutination responses. It is common for non-responding fish to be present in a population (Bricknell *et al.*, 1997). Some fish do appear to be non-responders but they are few. This is probably because the antigen was a commercial vaccine which will include an adjuvant thereby decreasing the numbers of non-responders.

Theoretically, triploids may be disadvantaged in comparison to diploids in respect of producing an antibody response. This is because

1. Triploids have a lower surface area to volume ratio and therefore the process may be rate limited
2. Triploids have fewer cells in total therefore a triploid may have less cells available with the ability to produce antibody (B cells). In addition a triploid would have an overall lower surface area, resulting in the potential for a triploid to have less antigen receptors in total.

Despite this, the triploids exhibit a higher agglutinating response than the diploids. This could be because the differences in cell size do not create a disadvantage for a triploid, but instead each antibody producing cell (plasma cell) has a larger cytoplasmic volume and can produce more antibody. In addition, although the

increase in cell size results in the potential for processes to be rate limited, it may also give an individual cell a greater antibody secreting potential.

8: An investigation of family variation of some non-specific, humoral immune parameters in diploid and triploid Atlantic salmon

8.1 Introduction

8.1.1 Genetics and disease resistance

It is increasingly evident from the literature that the ability of fish to resist pathogens has a high heritability. This results in species to species, strain to strain and individual to individual variation in disease resistance. However, this resistance itself may vary according to the pathogen in question.

Many authors have shown that individual fish from the same population have different abilities to resist bacterial infections (Beacham and Evelyn, 1992; Gjedrem and Gjoeen, 1995; Nilsson and Holmgren, 1992).

8.1.2 Genetics and immune parameters

Physiological and/or biochemical mechanisms that confer resistance in fish, can have a genetic basis. For example, bactericidal activity has been related to heritable resistance to *Aeromonas salmonicida* in rainbow trout (Hollebecq and Michel, 1989).

Antibody responses to antigens in outbred fish are often reported to be variable. This variability of specific antibody responses is reduced in homozygous fish (produced from self-fertilisation or gynogenesis). However, homozygosity also reduces antibody diversity (Desvaux *et al.*, 1987; Wiegertjes *et al.*, 1995).

T cell responses are regulated by a highly polymorphic set of genes called the major histocompatability complex (MHC). The extensive polymorphism of MHC genes means that many different alleles exist within a population, differing in their ability to bind different antigenic epitopes. This affects antigen presentation, T cell recognition and these in turn affect T cell responses. Wiegertjes (1995) has also

shown evidence of an association between MHC class II genes and the magnitude of antibody responses in carp.

Other parameters are known to have a genetic basis for variation without it being known how this affects disease resistance directly. Variation in serum hemolytic activity of Atlantic salmon appears to be genetically controlled (Roeed *et al.*, 1992) and genetic polymorphism of the complement component C3 has been shown in fish (Yano, 1996).

8.1.3 Influences on genetic expression

Genotype

The genotype is the genetic 'make-up' for a particular gene and is a combination of 2 alleles in a diploid situation.

In a diploid situation there are 2 sets of chromosomes and each will contain an allele for a particular characteristic. Each allele may be either 'dominant' or 'recessive'; a dominant allele is the allele which determines the phenotype of an organism if the organism is heterozygous (ie. has 1 dominant and 1 recessive allele).

In a triploid situation there are 3 sets of chromosomes and therefore 3 alleles for each gene. However, it is not known how this affects the phenotype. There may be 2 theoretical situations:

1. All 3 alleles function. This would result in a greater number of potential outcomes, and therefore more heterozygosity and more individual variation.
2. Only 2 alleles function. The 3rd allele may not exert an influence or only exert an influence under certain (unknown) conditions. It may be that the choice of the 'non-operative' allele is totally random and differs from cell to cell.

Environment

A phenotype is influenced not only by the genotype but also by environment. Environment may influence which genes are 'switched on' and also how those genes are expressed.

8.1.4 Aims of this experiment

Results from Chapter 3 found that some differences in non-specific humoral immune parameters may occur between sibling diploid and triploid all female Atlantic salmon. However, it seemed that these differences were not always consistent for different sibling groups of fish. It was therefore considered that possible family differences may account for such variation and this was the subject of this investigation.

8.2 Materials and Methods

8.2.1 Experimental Design

Serum samples were collected from sibling diploid and triploid Atlantic salmon from three families of the same age (one sea winter). These fish were reared and maintained at Matre Havforskningsinstitut, Norway. Water temperature was approx. 12.5 °C at the time of sampling. These families were designated a code to describe the individual mother and father. The letter was attributed to the father and the number to the mother. The families were therefore coded as follows: J20; O18; I18. Families J20 and I18 were mixed sex with milt being derived from a normal male. Family O18 was an all-female population with the milt being derived from a sex-reversed female. Therefore families O18 and I18 were half-siblings. The average weight of the sampled fish was 89.1g, 87g and 82.3g for families J20, O18 and I18 respectively. As described in Chapter 2, differential leucocyte counts and measurements of leucocyte sizes were made from blood smears collected from these fish.

Six groups were sampled; diploids and triploids from each family. The sampling regime is outlined in Table 8.1.The fish were anaesthetised with metamidate and blood sampled from the caudal vein with needles and syringes. The blood was placed into centrifuge tubes, allowed to clot and centrifuged to extract serum. The serum was aliquoted and stored at -80°C. Blood smears were also taken at the time of sampling, air dried, fixed as soon as possible in 70% methanol, stained with

Hema Gurr (BDH) and examined by light microscopy (x100 oil immersion) in order to confirm ploidy.

The serum samples were used to determine serum protein levels, alternative pathway complement activity, and serum iron with-holding activity (iron content (TI), unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC) and percentage serum transferrin saturation (% saturation)).

Sex was determined by visual examination of the gonads.

8.2.2 Assays

The methodologies for the assays used in this experiment are detailed in Chapter 3.

8.2.3 Statistical Analysis

Three-way analysis of variance was carried out using GENSTAT.

8.3 Results

There were no significant differences between male or female fish for any of the parameters. Ploidy and family differences, and their interactions, varied for each parameter.

Table 8.1 shows the individual data, means and standard errors for length, weight, ACH50, TI, UIBC, TIBC, percentage saturation and serum protein levels for diploids and triploids from each family.

Length was not significantly different for ploidy comparisons ($p=0.126$) but was highly significantly different between families ($p<0.001$) and there was a strong ploidy/family interaction ($p=0.008$).

There were no significant ploidy differences in complement activity ($p=0.978$), but once again there were family differences ($p<0.001$). This is shown in Figure 8.1.

Serum protein levels (see Figure 8.2) and TI (see Figure 8.3) both exhibited significant differences between diploids and triploids but not between families. The scale of difference between diploids and triploids varies between families and this is reflected statistically with a significant ploidy/family interaction ($p<0.001$).

Percentage transferrin saturation did not significantly vary between ploidy ($p=0.773$) or family ($p=0.076$).

For UIBC and TIBC, there were significant differences between ploidy and family but there was no interaction between ploidy and family. Therefore, the differences between diploids and triploids were not influenced in anyway by family for these parameters.

Table 8.1 Length, weight, serum protein concentration, alternative pathway complement activity and iron with-holding activity (TI, UIBC, TIBC and percentage saturation) of diploid and triploid siblings from 3 different families, showing mean, standard error and number of observations.

		J20		O18		I18	
		diploids	triploids	diploids	triploids	diploids	triploids
Length	Mean	19.32	19.23	18.5	19.85	18.18	18.93
	Std Err	0.19	0.13	0.25	0.46	0.20	0.28
	No. Obs	30	30	15	15	30	30
Weight	Mean	90.95	87.31	76.41	97.75	77.69	86.89
	Std Err	2.65	1.95	3.43	6.33	2.64	3.71
	No. Obs	30	30	15	15	30	30
Protein	Mean	49.57	36.96	31.45	46.63	41.54	37.00
	Std Err	2.45	1.09	1.68	3.04	1.45	1.61
	No. Obs	30	29	15	14	29	28
ACH50	Mean	68.82	49.89	115.62	159.39	54.87	46.67
	Std Err	16.93	2.25	12.31	15.11	4.18	3.47
	No. Obs	28	30	15	15	30	30
TI	Mean	2.74	2.09	3.14	1.94	2.39	2.63
	Std Err	0.19	0.13	0.29	0.28	0.12	0.14
	No. Obs	29	29	14	14	27	26
UIBC	Mean	6.13	5.71	5.52	3.28	6.21	4.75
	Std Err	0.44	0.37	0.46	0.44	0.43	0.29
	No. Obs	28	30	15	10	26	30
TIBC	Mean	8.93	7.73	8.53	4.96	8.56	7.36
	Std Err	0.49	0.40	0.54	0.59	0.51	0.37
	No. Obs	28	29	14	10	26	26
% sat	Mean	32.44	28.14	37.44	33.27	28.38	36.35
	Std Err	1.93	1.71	2.74	4.87	1.27	1.55
	No. Obs	28	29	14	10	26	26

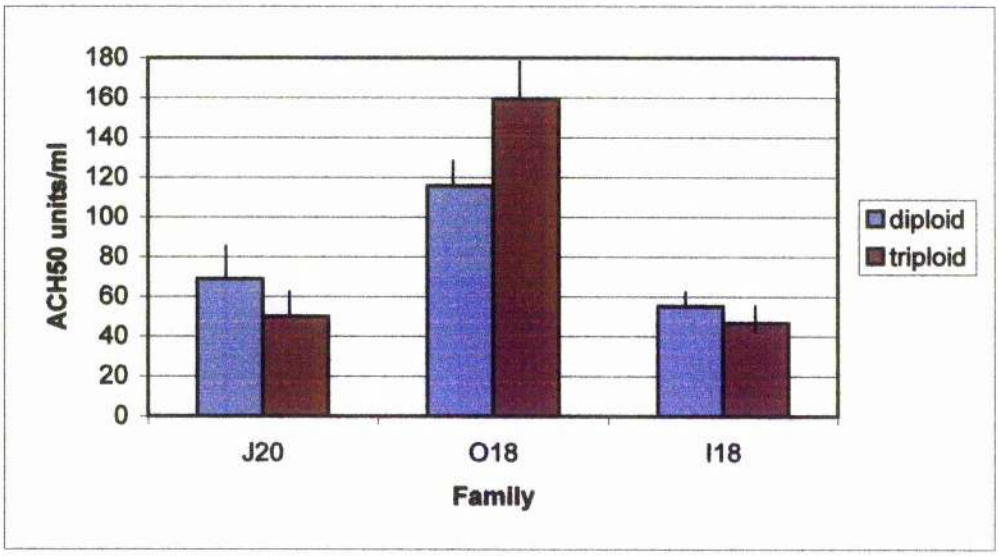


Figure 8.1 Alternative pathway complement activity of diploid and triploid Atlantic salmon from 3 different families.

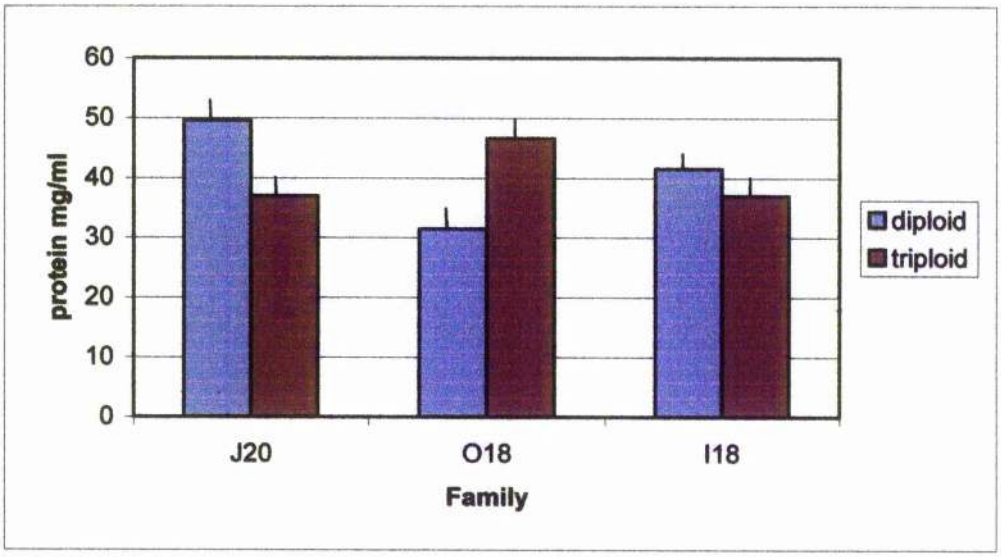


Figure 8.2 Serum protein concentrations of diploid and triploid Atlantic salmon from 3 different families.

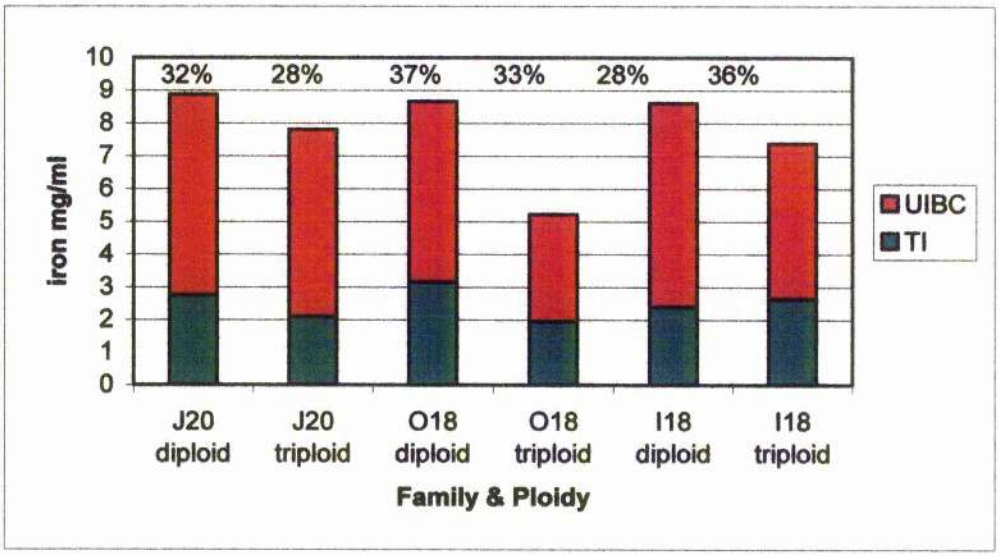


Figure 8.3 Serum iron with-holding activity of diploid and triploid Atlantic salmon from 3 different families, showing TI (green), UIBC (red), TIBC (red+green) and percentage saturation.

8.4 Discussion

8.4.1 Ploidy variation

Alternative pathway complement activity did not vary significantly between diploids and triploids which agrees with the literature and Chapter 3. However, serum iron with-holding can (but not always) differ. From examination of the 'standard error' (see Table 8.1) there are no consistent differences between diploids and triploids. On occasions when differences do occur, diploids often have higher 'standard error'. This suggests that, within a family group, triploids do not have a wider range for the parameters than diploids, therefore implying that they do not exhibit greater heterogeneity of the phenotype.

8.4.2 Family variation

For some parameters it could be concluded that family differences were of greater significance than ploidy differences (for example ACP activity). For other parameters it was found that ploidy differences varied according to family (some iron with-holding parameters).

This suggests that although some non-specific immune parameters do vary between diploids and triploids, family effects are evident and often of greater significance.

Since the environment in which the different families were reared did not differ, it may be that the possession of an extra chromosome affects the genotype of triploid fish. As described in Chapter 8.1.3, there are 2 theoretical consequences of possessing an extra chromosome

1. All 3 alleles function resulting in a greater number of potential genotypes
2. Only 2 alleles function with the 3rd allele not exerting an influence or only exerting an influence under certain (unknown) conditions.

From the results of this study, it is possible that the 1st potential outcome is the case. However, this does not agree with the information drawn from the examination of 'standard error' which shows that triploids do not have consistently higher standard errors when compared to sibling diploids. This suggests that triploids do not show

greater heterogeneity of the phenotype. Nothing is known of gene-dose relationships in lower vertebrates and these would need to be studied in detail before such conclusions can be drawn. However, triploid fish provide the perfect research tool for such a study and may prove in the future to be invaluable for this reason.

An interesting result is the comparison of the half-sibling families O18 and I18. When large family differences occur in this study, it is often because the family O18 is different to the other families. From examination of the 'standard error', this is also the family which shows the widest range of results for a parameter (for diploids and triploids). O18 is an all-female family and has a different 'father' to I18 in that a sex-reversed female was used to provide the milt for fertilisation. From this study it seems that the manipulation of sex to produce all-female families has a greater impact on immune parameters than triploidy.

9. Investigation of the effect of chronic stress on the disease susceptibility of diploid and triploid salmon

9.1 Introduction

9.1.1 Introduction

Intensive aquaculture of fish often necessitates the use of various handling and transportation techniques that can result in increased morbidity or mortality. It is well known that stress increases the susceptibility of fish to disease (Barton and Schreck, 1987; Peters *et al.*, 1988; Pickering and Pottinger, 1987). The stress-activated mechanism of immunosuppression in fish is not completely understood but appears to be mediated via endocrine pathways (Barton and Iwama, 1991). The relationship between stress and infection in mammals has been well documented and, in brief, shows that stress induced under laboratory conditions (exercise, restraint, isolation, temperature) make mammals more susceptible to primary infection with bacteria and viruses.

Stress can be defined as the reaction of the body to deleterious forces (stressors). Stress results in a cascade of events that typically involves the release of catecholamines and cortisol. There are 3 levels of stress responses; primary, secondary and tertiary. These are described below.

9.1.2 Stressors

Stressors can be classified in several ways. For example, acute versus chronic stressors, or artificial versus natural. In the classification of acute versus chronic stress, acute stressors are short lived but high level stressors (eg. handling) whereas chronic stressors are of a lower intensity but longer term (eg. non-optimal oxygen levels or predatory stressors) (Barton and Iwama, 1991). Prior exposure to stressors can affect the stress response. Chinook salmon experienced cumulative physiological responses to each subsequent stressor event (Barton *et al.*, 1986).

Temperature appears to be a major factor affecting stress responses. Barton and Schreck (1987) found that temperature affected the carbohydrate stress response rather than the inter-renal stress response in Chinook salmon. However, temperature stress (also referred to as thermal shock) does not necessarily increase susceptibility to disease. Rainbow trout exposed to a temperature shock did not have an increased susceptibility to *Flexibacter columnaris*, and on some occasions it actually increased resistance (Barton and Iwama, 1991).

9.1.3 Physiological effects of stress

The stress responses of fish have been classified by Barton and Iwama (1991) as primary, secondary and tertiary responses, according to the level of organisation of the response. Many of these responses are used as quantitative indicators of stress in fish.

Primary stress responses occur quickly and can be short-lived. These include increases in plasma catecholamines, corticosteriods and ACTH (Barton and Iwama, 1991).

Secondary and tertiary stress responses result directly or indirectly from the influence of catecholamines and cortisol (Schreck, 1996). Secondary responses are characterised by certain parameters which can be classified into further categories; metabolic, haematological, hydromineral and structural responses. They do not occur as quickly as primary responses but they are evident before tertiary responses. Secondary responses include increased plasma glucose and protein, changes in haematocrit, erythrocyte counts and lymphocyte:erythrocyte ratios. Tertiary responses include changes in growth rates and disease resistance (Barton and Iwama, 1991).

9.1.4 Effect of stress on the immune system

Stress is often considered to have an immunosuppresive effect. Reduced immune responses are often associated with elevated glucocortoid and ACTH levels. The administration of exogenous cortisol has been shown to produce

immunosuppression similar to that caused by certain stressors (Ellsaesser and Clem, 1986). Elevated levels of cortisol may inhibit secretion of cytokines involved in the differentiation of lymphocytes (Kaattari and Tripp, 1987). Serum lysozyme activity is also increased in stressed fish (Fevolden *et al.*, 1994).

However, although handling and confinement have been shown to be immunosuppressors (Ellsaesser and Clem, 1986; Schreck, 1982; Yin *et al.*, 1995) temperature stress has a different effect. Higher than ambient water temperatures (within the physiological range of the fish) can enhance some immune functions. For example, lymphocyte proliferation of tench (*Tinca tinca*) was highest in summer, but conversely, alternative pathway complement activity and phagocyte functions were elevated at winter temperatures (Schreck, 1996).

Stressors are likely to affect more than just the immune system and may upset other homeostatic functions. This has been demonstrated in channel catfish where an acute decrease in water temperature leaves the fish immunocompromised but also significantly slows mucus cell migration through the dermis (Bly and Clem, 1991; Bly *et al.*, 1992). The combination of these effects resulted in a higher incidence of saprolegnia.

Different forms of acute stress result in leucopenia (lowering of blood leucocyte numbers), lymphopenia (lowering of blood lymphocyte numbers) and neutrophilia (increase of blood neutrophil numbers). Ellsaesser and Clem (1986) also claim that the remaining lymphocytes have a much reduced immunological function since they no longer respond *in vitro* to the mitogens LPS and Con A, nor do they undergo primary anti-hapten antibody responses to either T-independent or T-dependent antigens.

9.1.5 Stress and triploids

Although experiments have shown that triploids should not be restricted (in terms of growth, disease resistance etc) under optimal conditions in comparison to diploids, sub-optimal conditions may affect these parameters and thereby

potentially cause higher triploid mortality rates under farming conditions (Johnson *et al.*, 1986; Ojolick *et al.*, 1995; Quillet and Gaignon, 1990; Yamamoto and Iida, 1994). Since surface area to volume ratios are reduced as cell size increases, the ability of triploid cells to respond to hormonal or neural stimuli may be diminished (Benfey, 1991). Furthermore the absence of gonadal development affects steroidogenesis and pituitary function (Benfey *et al.*, 1989) and possibly also intermediary metabolism, thereby also affecting the stress response. There is anecdotal evidence in the literature that triploid fish may be more susceptible to chronic stress than diploids (Johnson, *et al.*, 1986; Ojolick, *et al.*, 1995; Quillet and Gaignon, 1990; Yamamoto and Iida, 1994). However, there is no difference in their toleration of acute stress (Biron and Benfey, 1994).

9.1.6 Aims of this experiment

It is hypothesised that exposure to chronic stressors may lower the immunocompetence of triploids and when coupled with an infection, the disease resistance of a triploid fish may become lower than that of a diploid sibling. The aim of this experiment was to expose diploid and triploid Atlantic salmon to a chronic stressor daily over a period of several weeks, and then challenge with disease. Haematological, stress-related and non-specific humoral defence parameters were determined before and after the stress exposure to quantify the level of the stress and to monitor the effect of stress on constitutive defence parameters in diploids and triploids.

The aim of studying fish blood was to determine numbers and proportions of cells such as erythrocytes and leucocytes in order to compare the response of the immune system before and after a stress event in diploid and triploid fish and also to indicate any metabolic secondary responses to stress. Plasma glucose and protein levels were also determined to indicate a stress response.

9.2 Materials and Methods

9.2.1 Experimental design

All-female, Atlantic Salmon of the same family were maintained at ambient temperature (mean 10°C) in flow-through, seawater in 1 m^3 tanks. The experimental regime is outlined in Table 9.1.

On day 0, 10 diploids (mean weight :32 g, length : 13.96 cm) and 10 triploids (mean weight : 44 g, length : 15.51 cm) were sampled for serum (as described in Chapter 3.2), blood smears, total cell counts and haematocrits (as described in Chapter 2).

Diploid and triploid salmon were separated into 8 groups of 35 fish after the Day 0 sampling: 2 tanks with diploid fish (diploid unstressed), 2 tanks with diploid fish and an adult salmon, 500-600 g (diploid stressed), acting as predator, and the same distribution for the triploid fish (triploid unstressed and triploid stressed).

Two weeks after first exposure to the stressor, 5 fish were sampled from each tank. The predator stress was removed and the remaining fish challenged with *Aeromonas salmonicida.*

Table 9.1 Experimental regime for baseline (B), stressed (S) and unstressed (unS) diploid (D) and triploid (T), all-female Atlantic salmon

Group	Ploidy	Sampled on	Stress Treatment (Yes/No)	Disease Exposure On Day 14
DB	Diploid	Day 0	N	-
TB	Triploid	Day 0	N	-
DS	Diploid	Day 14	Y	*A. salmonicida*
TS	Triploid	Day 14	Y	*A. salmonicida*
DunS	Diploid	Day 14	N	*A. salmonicida*
TunS	Triploid	Day 14	N	*A. salmonicida*

9.2.2 Non-specific assays

Alternative pathway complement activity (described in Chapter 3.2), iron with-holding activity (described in Chapter 3.2) and macrophage respiratory burst activity (described in Chapter 6.2) were used to assess constitutive defence mechanisms.

9.2.3 Stress parameter assays

Glucose

This assay was used to determine the concentration of glucose in the samples of serum. A glucose assay kit (Sigma) was used.

One ml of glucose reagent was added to 10 µl of blank (distilled water), standard or test (serum) in 1.5ml UV grade, disposable cuvettes. Solutions were mixed by slow inversion and incubated at room temperature for 10 min. At this time, the reaction has finished and is stable for 1 hour. However, all samples were read immediately after 10 min incubation. The optical density (OD) was then read at 340 nm on a spectrophotometer.

The concentration of glucose for each sample was determined from a standard curve and expressed as mg per ml of serum.

9.2.4 Disease challenge

After the period of stress, the fish were challenged with *Aeromonas salmonicida* to compare the post-stress disease resistance of diploids and triploids.

Aeromonas salmonicida MT004 was cultured on tryptone soy agar (TSA) at 22°C for 2 days and then harvested into phosphate buffered saline. Bacteria were added to the tanks to produce a final concentration of 10^5 cells/ml. The water supply of the tanks was switched off and the water aerated for 24 hours. The fish were starved for 24 hours prior to challenge and during the 24 hour bath challenge. Mortalities were monitored daily, collected, recorded and dead fish stored at –20°C.

Swabs from kidneys of all dead fish (defrosted) were made onto TSA to ensure cause of death.

9.2.5 Statistical analysis

Results were analysed by two way analysis of covariance (using weight as the covariant) on GENSTAT.

9.3 Results

Table 9.2 shows a summary of the mean responses for each group.

9.3.1 Haematology

Haematocrits were recorded only for the stressed and unstressed fish on Day 14. Day 0 samples were not collected due to technical problems. There are no statistical differences between stressed or unstressed fish, diploids or triploids.

Erythrocyte and leucocyte percentages in peripheral blood were measured for diploids and triploids baseline (Day 0), stressed and unstressed. Erythrocyte percentages do not significantly change for ploidy or stress. Leucocyte percentages do not differ between ploidy, but there are differences between baseline, stressed and unstressed groups.

9.3.2 Plasma assays

Glucose significantly differs between diploids and triploids at Day 0 with diploids having higher glucose levels. This is also the case at Day 14 within the stressed groups. However, there are no significant differences between diploids and triploids at Day 14 in the unstressed groups. There are no significant differences between the baseline groups (Day 0) and the stressed groups.

There are no significant differences in plasma protein levels between diploids and triploids within a treatment group. However, the unstressed groups have

higher protein levels than the baseline groups, and the stressed groups have higher protein levels than either of these.

There are no significant differences for any of the iron with-holding parameters (TI, UIBC, TIBC and % saturation) between diploids and triploids in stressed or unstressed groups. There are also no significant differences between the stressed or unstressed groups. However, (although the data for diploids are missing) it appears that iron with-holding levels differ between the baseline groups and stressed and unstressed groups, with all groups sampled at day 14 having increased total iron content and iron binding capacity.

There were no significant differences in complement levels between diploids and triploids in any group. However, all groups sampled on Day 14 (stressed and unstressed) have higher ACH50 than fish sampled at Day 0.

9.3.3 Cellular assays

There were no significant differences in respiratory burst activity between ploidy or between stress treatments.

Table 9.2 Mean responses of diploid and triploid, baseline (DB, TB), stressed (DS, TS) and unstressed groups (DunS, TunS) (* indicates data that is missing due to insufficient serum or loss during processing).

	DB	TB	DunS	TunS	DS	TS
Weight g	32.30	44.40	33.32	47.28	38.28	39.92
Length cm	13.96	13.43	14.60	15.20	14.20	14.30
Haematocrits %	*	*	49.80	43.57	47.57	51.00
Erythrocytes %	95.54	95.33	95.93	95.74	95.44	95.73
Leucocytes %	4.45	4.65	4.07	4.06	4.62	4.27
Complement ACH50 units/ml	21.12	15.22	47.00	63.94	67.04	43.95
Total serum iron µg/ml	*	190.76	334.27	332.87	326.73	342.63
Serum UIBC µg/ml	*	183.80	342.43	337.48	382.93	328.36
Serum TIBC µg/ml	*	374.56	676.71	670.36	709.66	670.99
% saturation	*	54.49	47.97	48.29	45.35	49.69
Serum glucose g/l	12.51	10.77	11.75	11.28	12.09	9.33
Respiratory burst OD/ 10^5 cells	0.005	0.013	0.011	0.045	0.07	0.028

9.3.5 Challenge

As shown in Figure 9.1, the triploid fish seem to survive better than diploid fish whether they are stressed or unstressed. However, because of the low numbers of fish used in each group, this is not a significant result. There are no differences in total mortality between stressed or unstressed fish.

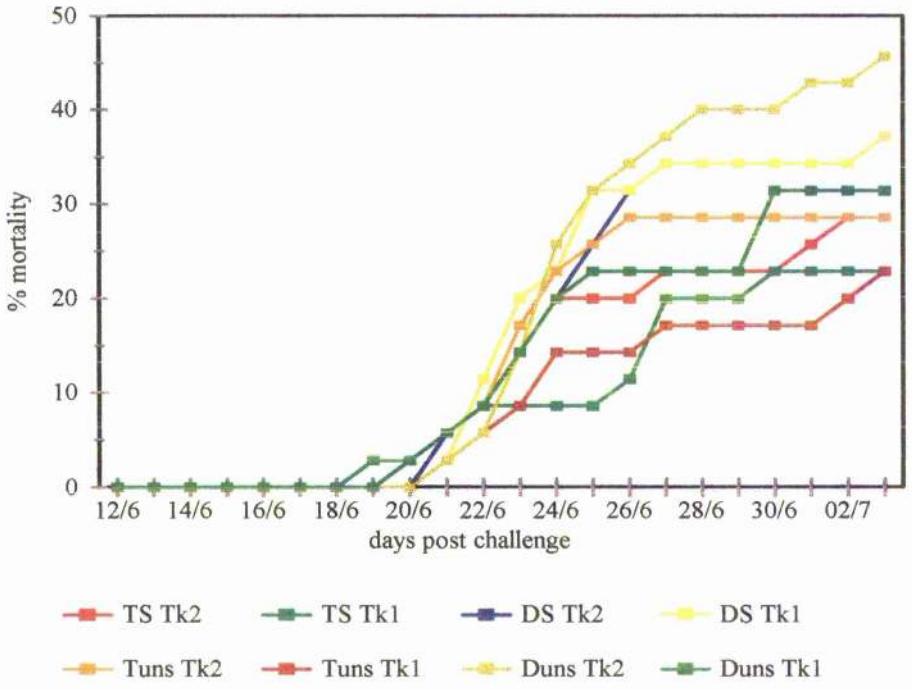


Figure 9.1 Cumulative percentage mortality of diploid and triploid, Atlantic salmon, previously exposed to stress or non-stressed.

9.4 Discussion

9.4.1 Stress response

An increase in mortality rate of Atlantic salmon can be produced by the synergistic effect of osmotic stress and predator stress (Jarvi, 1989). The predator stress was shown to elevate cortisol and glucose in the 'prey' fish. This is a similar response to social interactions (eg, hierarchial dominance) (Barton and Iwama, 1991). However, these deaths were not caused by disease. It is hypothesised by Jarvi (1989) that the increase in cortisol caused elevated adrenaline levels which in turn disturbs normal glucose metabolism and leads to acidosis, by causing an increase in the concentration of lactic acid in the blood and tissues (Schreck, 1982). From this information, it could be hypothesised that the reported increased mortality of triploids in culture, often associated with their increased sensitivity to stress (Benfey, 1997), is not due to stress induced immunosuppression and therefore death by disease, but instead this mortality is due to an increased susceptibility to acidosis due to the triploid trait of fewer and larger cells.

Stress responses monitored in this study are serum protein and glucose levels (which would be expected to increase in response to stress). From Table 9.1 it can be seen that there is a trend for these parameters which could demonstrate a stress response between day 0 and day 14. These responses show an increase in stress response between the unstressed and stressed groups. However, the unstressed groups show higher stress levels than the baseline groups. This could be attributed to problems with water supply. At day 12 a major problem occurred at the fish cultivation unit which resulted in no water flow for approximately 15 hours. Because the stocking density for this experiment was purposely low, the fish survived this water stoppage. However, it appears from these results that all fish in all groups experienced stress. But interestingly, the additional stress of a predator seems to have caused a slightly higher stress response.

The magnitude and pattern of change with time of stress responses between Day 0 and Day 14 unstressed fish did not differ between diploids and triploids. This

is in agreement with stress responses of diploid and triploid brook trout (Biron and Benfey, 1994). However, Biron and Benfey (1994) report the possibility that secretion and/or clearance rates of cortisol may differ between the two groups. However, another report shows that increases in plasma cortisol levels of diploid and triploid Atlantic salmon did not differ when the fish were exposed to confinement stress (Sadler *et al.*, 1998). Both diploids and triploids displayed a significant primary endocrine response to stress. Sadler *et al.* (1998) also found that plasma glucose and lactate increased as a result of confinement stress in Atlantic salmon although no differences were detected between diploids and triploids. Plasma protein did not change in any groups in response to stress. These results are in close agreement with those of Biron and Benfey (1994) following acute stress. This suggests that the primary endocrine response to stress in triploids follows the typical salmonid response (Barton and Iwama, 1991; Pickering and Pottinger, 1987).

9.4.2 Stress and immune parameters

The results from this study did not show differences in non-specific humoral immune parameters between diploids and triploids within a treatment group. There were also no differences between treatments. This could be interpreted as demonstrating that there was no stress effect. However, as discussed above, other parameters suggest that a stress response did occur.

9.4.3 Stress and disease resistance

There were no differences in mortality of diploids and triploids exposed to *Aeromonas salmonicida*, or differences between stressed and non-stressed fish.

The comparative susceptibility of triploid and diploid fish to disease is still debated and reports in the literature do not demonstrate consistent results (see Chapter 1). There are no reports in the literature on the interaction between stress and disease in triploid fish.

There does appear to be evidence that stress can increase susceptibility to disease. Many fish pathogens are ubiquitous. However, interactions between fish and pathogens can be harmless but under culture conditions the addition of stress from the physical, chemical and biological conditions can cause these otherwise harmless pathogens to become pathogenic. Many of the physiological mechanisms that allow infections to become established and to spread when fish are stressed are not thoroughly understood, but it appears that both the specific and non-specific immune systems are affected.

Various non-specific parameters (for example complement, phagocytosis) are adversely affected when fish are stressed (Ellsaesser and Clem, 1986). Effects of stress on other important functions such as debris removal during tissue repair or mucus cell migration through the dermis (Bly and Clem, 1991; Bly, *et al.*, 1992) may also be involved in allowing infections to become established.

10: General Discussion

10.1 The effect of cell size on the immune system

10.1.1 Larger and fewer cells

Investigations into various haematological parameters were made in order to confirm the accepted conclusion that haematocrits do not differ between diploids and triploids, but that erythrocytes are larger and fewer in number (Benfey *et al.*, 1984; Biron and Benfey, 1994; Graham *et al.*, 1985; Small and Randall, 1989; Yamamoto and Iida, 1994). Only one previous report of these results in Atlantic salmon exists in the published literature (Benfey, *et al.*, 1984). Results in this thesis confirmed that haematocrits do not differ between diploids and triploids but that total blood cell numbers and erythrocyte numbers were lower in triploids.

Only one report on leucocyte sizes in triploid fish exists within the literature (Small and Benfey, 1987). This described the size of polymorphonuclear leucocytes (neutrophils) in Atlantic and Coho salmon. However, it is not clear from this report if these were peripheral blood neutrophils or not. No other leucocytes have been described in triploid fish. In this study an investigation into the sizes of peripheral blood leucocytes showed that these leucocytes are also larger in triploids and fewer in number. This was found to be true for 3 families of Atlantic salmon. It was also found that kidney macrophages were larger in triploids and observations of macrophage yields suggest that they are fewer in number.

The proportions of the different leucocyte populations did not differ between diploids and triploids. This was the case for 3 families of diploids and triploids. Neither did the proportion of the whole population that might be active at any one time (as shown by phagocytic activity) appear to differ between the ploidy types.

10.1.2 Cellular immune parameters

The consequence of larger but fewer leucocytes has not been fully investigated. Only 2 parameters were investigated; phagocytosis and intracellular respiratory burst activity. The altered cell volume to surface area does have consequences at the

individual cell level. Individual triploid kidney macrophages ingest more than diploid ones. This is consistent with their larger size. The factor of increase was a third and this would suggest that the increase in phagocytic capacity per cell will be balanced by having fewer cells in total.

Although it might be expected that the relative proportions and types of binding sites on the surface of macrophages would not differ between diploids and triploids, an individual triploid cell would presumably have more sites. This would possibly be in direct proportion to its greater surface area. Providing the concentration of substrate is not limiting (eg. Yeast availability in the phagocytosis assay), it would be expected that triploid cells would have higher activity levels for factors dependent on surface stimulation.

However, this did not seem to be the case for intracellular respiratory burst activity. This parameter is very dependent on membrane stimulation. Results from this thesis show that intracellular respiratory burst activity (an important killing mechanism) is not increased in triploids. If this were the case the total killing efficiency of the triploid non-specific, cellular immune system maybe compromised.

In addition to increased surface area per cell in triploids, they also have increased cell volume. However, they should have proportionally more cell volume to 'cope' with the increased rate of uptake of material than is necessary for their enlarged surface area. This is because, theoretically, cell volume would increase by one third whereas the surface area would increase by less. Considering this, it would therefore be expected that an individual triploid cell could process more material through its enlarged surface area and further process that material even more efficiently than a diploid cell by virtue of its increased volume. A triploid macrophage can process more by virtue of its increased surface area (shown by increased phagocytic index). It has an increased cell volume to store this extra material, but it does not seem to be more efficient in processing this material intracellularly when compared to a diploid cell (as shown by intracellular respiratory burst activity).

However, it is possible that the assay did not fully stimulate the triploid cells and therefore they did not respond to their full capacity.

In contrast, the evidence of Brodsky and Uryvaeva (1978) demonstrates that with increased ploidy, cell membranes become more indented. If this is the case, the total surface area of triploids may not be reduced. Consequently the stimulation of phagocytes and the resultant respiratory burst activity would not be reduced in triploid Atlantic salmon in comparison to diploids.

10.1.3 Humoral parameters

It is also possible to explain the differential hypoferraemic response and alternative complement changes seen following LPS injection. Triploids take longer to respond and then slightly longer to return to baseline levels, in comparison to diploids. This may cause them to be more susceptible for longer and is consistent with the commercial experience, namely that triploids suffer increased losses particularly when faced with multiple insults. This may be due to increased cell volume causing the process to be rate limited (eg. transfer distances are increased). This may mean that a triploid cell is disadvantaged, because although it is able to take in more of a substance, it can only process this substance at the same rate as a diploid cell. When considered in parallel with the fact that there are fewer individual triploid cells contributing to the whole effort, triploids may be at a disadvantage.

10.2 Consequences of a 3rd chromosome set

10.2.1 Variability

The triploid cell contains one third more genetic material by virtue of the extra maternal chromosome. Triploids are therefore potentially more heterozygous, but this potential heterozygosity may not always be expressed. Very little is known about gene dose relationships in lower vertebrates. If it is assumed that a gene dose relationship does exist in fish, a population of triploid salmon might be expected to express a wider range of variability than a comparative diploid population. In a trait controlled by a simple dominant/ recessive allelic mechanism (A/a), the doubly

dominant constitution (AA) and the doubly recessive combination (aa) can be viewed as the extremes of the range of expression. Other combinations (Aa or aA) would be intermediate. If the extra genetic material in triploids is expressed, two new extremes are possible (AAA and aaa) together with additional intermediates (Aaa and aaA). Although most traits are not controlled by a single gene locus but are instead polygenic, the same kind of phenomenon may apply.

Although for many biological parameters there is no difference between diploid and triploid fish, there do appear to be differences in the distributions of the populations. For example, Hanson (pers comm) has found that length, weight and growth performance are not significantly different between diploid and triploid Atlantic salmon, but when comparing frequency distributions the best and worst performers are always triploid.

The possession of 3 chromosomes by a triploid could potentially increase the variability within a population. From the results of Hanson (pers comm), it seems that this may be so. However, studies of frequency distributions for some factors within this study did not find such a pattern. Triploids were not consistently the best and worst performers.

10.2.2 Non-specific, humoral parameters

Results from Chapter 2 found that some differences in non-specific, humoral immune parameters may occur between diploid and triploid Atlantic salmon but those differences were not consistent between groups. This led to an investigation into family variation (Chapter 6).

For some parameters it could be concluded that family differences were of greater significance than ploidy differences. For other parameters it was found that ploidy differences varied according to family. For yet other parameters, ploidy differences existed which were in no way influenced by family. This suggests that although some non-specific immune parameters do vary between diploids and triploids, family effects are evident and often of greater significance.

Complement did not vary between diploids and triploids which agrees with the literature. However, serum iron with-holding can (but not always) differ. Comparison with the literature is difficult because little work on the comparative immunology of diploid and triploid fish has been done. No results are presented in the literature comparing immune parameters in diploid and triploid Atlantic salmon.

10.3 General immunocompetence of triploid Atlantic salmon

The comparison of different studies and the arrival at unifying conclusions is confounded by the variation of a number of factors which are known to modulate immune responses. These include species, age and size of fish and environmental factors such as temperature, water quality and nutrition. Fish are the largest group of vertebrates and it can be very misleading to generalise about any aspect of their biology. In addition, the immune system is one of many physiological processes and at different times within an individuals life the requirement for resource partitioning will lead to fluctuations in the resources committed to immune defences.

The immune system is also highly complex one and only a few aspects have been investigated in fish in general, with even less often known about a particular species. Many of the immune factors studied can function independently. However, fish like mammals have a well-developed lymphoid system with a comparable level of immunological intricacy and possess an intertwined system of cellular and non-cellular parameters and interconnecting specific and non-specific system.

Most of the immunological factors measured in this study were humoral, but these factors would originally be produced by cells. However, serum concentrations are similar in most cases between diploids and triploids. This suggests that although there are fewer cells to produce these factors, the cells are collectively producing the same amount ie. each triploid cell is producing more.

Sadler *et al.* (1998) have found that the mean red cell RNA concentrations were similar for diploid and triploid Atlantic salmon. If the concentrations are the same, a

triploid cell will have a 1/3 more RNA than a diploid because it is 1/3 larger. If it has 1/3 more RNA it must be able to produce 1/3 more protein. Therefore protein synthesis rates could be greater for triploid cells which would explain the similarity in serum levels in diploids and triploids, for factors which are cellular derived. There are no other data reported in the literature comparing RNA content for diploid and triploid Atlantic salmon or other fish species.

10.4 Disease resistance of triploid Atlantic salmon

Overall, studies of disease factors in this thesis did not demonstrate any definitive or consistent inferior performance of triploids. This is in agreement with the results of the disease trial executed within Chapter 9 in which no difference in disease susceptibility between diploid and triploid Atlantic salmon, was found. Trials carried out by Glette (unpublished) are in agreement with this. Reports of comparative diploid and triploid Atlantic salmon and other fish species do not always agree with this finding. Some authors report increased susceptibility in triploids. However, as stated above, there are many reasons why it is difficult to compare these different studies with the data reported in this thesis.

The data found in this thesis regarding disease susceptibility are in contrast to the results of commercially grown populations of triploid Atlantic salmon (especially in cages) and with the experience in Scotland and Canada which sugggests that triploids are more susceptible to disease. It may be hypothesised that this is because in commercial culture, the additive effects of several sub-acute factors are more likely to occur than under experimental conditions. The overall 'comfort zone' of triploids (ie. their tolerance of sub-acute conditions) may be narrower than that of diploids due to the physiological differences caused by increased cell size and decreased cell numbers. However, an investigation into this (Chapter 9) was unable to demonstrate this experimentally.

Bibliography

Absher, M. (1973). Haemocytometer counting. In *Tissue Culture; Methods and Applications*, pp. 395-397. Edited by P. F. Kruse & M. K. Patterson: Academic Press.

Afonso, A., Lousada, S., Silva, J., Ellis, A. E. & Silva, M. T. (1998). Neutrophil and macrophage responses to inflammation in the peritoneal cavity of rainbow trout *Onchorynchus mykiss*. A light and electron microscopic cytochemical study. *Diseases of Aquatic Organisms* **34**, 27-37.

Afonso, A., Silva, J., Lousda, S., Ellis, A. E. & Silva, M. T. (1998). Uptake of neutrophils and neutrophilic components by macrophages in the inflamed peritoneal cavity of rainbow trout (*Onchorynchus mykiss*). *Fish & Shellfish Immunology* **8**, 319-338.

Ainsworth, A. J. (1992). Fish Granulocytes: morphology, distribution and function. *Annual review of Fish Diseases* , 123-148.

Alexander, J. B. & Ingram, G. A. (1992). Non-cellular, non-specific defence mechanisms of fish. *Annual Review of Fish Diseases* , **2** 249-279.

Aliah, R. S., Inada, Y., Yamaoka, K. & Taniguchi, N. (1991). Effects of triploidy on hematological characteristics and oxygen consumption in ayu. *Nippon Suisan Gakkaishi.* **57**, 833-836.

Aliah, R. S., Yamaoka, K., Inada, Y. & Taniguchi, N. (1990). Effects of triploidy on tissue structure of some organs in ayu. *Nippon Suisan Gakkaishi.* **56**, 569-575.

Allen, S. K. (1983). Flow cytometry: assaying experimental polyploid fish and shellfish. *Aquaculture* **33**, 317-328.

Anon. (1994). Report on Disease Prevention for the New Brunswick farmed salmon industry research consortium. : University of New Brunswick.

Arai, K., Matsubara, K. & Suzuki, R. (1993). Production of polyploids and viable gynogens using spontaneously occurring tetraploid loach, *Misgurnus anguillicaudatus*. *Aquaculture* **117**, 3-4.

Avtalion, R. R., Weiss, E. & Moalem, T. (1976). Regulatory effects of temperature in ectothermic vertebrates. In *Comparative Immunology*, pp. 227-238. Edited by J. J. Marchalonis: Blackwell.

Barrett, A. J. & Starkey, P. M. (1973). The interaction of α_2-macroglobulins with proteinases. *Biochemical. Journal* , 709-724.

Barton, B. A. & Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteriods. *Annual review of Fish Diseases* **1**, 3-26.

Barton, B. A. & Schreck, C. B. (1987). Influence of acclimation temperature on inter-renal and carbohydrate stress responses in juvenile chinook salmon (*Onchorynchus tshawytscha*). *Aquaculture* **62**, 299-310.

Barton, B. A., Schreck, C. B. & Sigismondi, L. A. (1986). Multiple acute disturbances evoke cumulative physiological stress responses in juvenile chinook salmon. *Transactions of the American Fisheries Society* **115**, 245-251.

Beacham, T. D. & Evelyn, T. P. T. (1992). Genetic variation in disease resistance and growth of chinook, coho and chum salmon with respect to vibriosis, furunculosis and bacterial kidney disease. *Transactions of the American Fisheries Society.* **121**, 456-485.

Benfey, T. J. & Sutterlin, A. M. (1984). Growth and gonadal development in triploid landlocked Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences* **41**, 1387-1392.

Benfey, T. J. & Sutterlin, A. M. (1984). The haematology of triploid landlocked Atlantic salmon, *Salmo salar*. *Journal of Fish Biology* **24**, 333-338.

Benfey, T. J. (1989). A bibliography of triploid fish, 1943 to 1988. *Canadian Technical Reports on Fisheries and Aquatic Sciences.*

Benfey, T. J. (1991). The physiology of triploid salmonids in relation to aquaculture. *Proceedings Of The Atlantic Canada Workshop On Methods For The Production Of Non Maturing Salmonids: February* , 73-80.

Benfey, T. J. (1998). The physiology and behaviour of triploid fishes. *Reviews in Fisheries Science* in press.

Benfey, T. J., Dye, H. E. & Donaldson, E. M. (1989). Estrogen-induced vitellogenin production by triploid coho salmon (*Oncorhynchus kisutch*), and its effect on plasma and pituitary gonadotropin. *General Comparative Endocrinology* **75**, 83-87.

Benfey, T. J., Sutterlin, A. M. & Thompson, R. J. (1984). Use of erythrocyte measurements to identify triploid salmonids. *Canadian Journal of Fisheries and Aquatic Sciences* **41**, 980-984.

Bezkorovainy (1987). Iron proteins. In *Iron and Infection*, pp. 27-67. Edited by J. J. Bullen & E. Griffiths. New York: John Wiley & Sons.

Bilinski, E., Boyce, N. P., Jonas, R. E. E. & Peters, M. D. (1984). Characterisation of protease from the myxosporean salmon parasite, *Henneguya salmonicida*. *Canandian Journal of Fisheries and Aquatic Sciences* **41**, 371-376.

Biron, M. & Benfey, T. J. (1994). Cortisol, glucose and haematocrit changes during acute stress, cohort sampling, and the diel cycle in diploid and triploid brook trout *(Salvelinus fontinalis* Mitchill). *Fish Physiology and Biochemistry* **13**, 153-160.

Blaxhall, P. C. & Daisley, K. W. (1973). Routine haematological methods for use with fish blood. *Journal of Fish Biology* **5**, 771-781.

Blaxhall, P. C. (1972). The haematological assessment of the health of freshwater fish: a review of selected literature. *Journal of Fish Biology* **4**, 593-604.

Bly, J. E. & Clem, L. W. (1991). Temperature mediated processes in teleost immunity: *in vitro* immunosuppression induced by *in vivo* low temperature in channel catfish. *Veterinary Immunology and Immunopathology* **34**, 365-377.

Bly, J. E., Lawson, L. A. & Clem, L. W. (1992). Temperature effects on channel catfish to a fungal pathogen. *Diseases of Aquatic Organisms* **113**, 155-164.

Bognor, K. H. & Ellis, A. E. (1977). Properties and functions of lymphocytes and lymphoid tissues in teleost fish. *Fish & Environment* **4**, 59-72.

Boulanger, Y. (1991). Performance comparison of all-female, diploid and triploid brook trout. *Proceedings Of The Atlantic Canada Workshop On Methods For The Production Of Non Maturing Salmonids: February* , 111-121.

Bowden, T. J., Butler, R., Bricknell, I. R. & Ellis, A. E. (1997). Serum trypsin-inhibitory activity in five species of farmed fish. *Fish & Shellfish Immunology* **7**, 377-385.

Bricknell, I. R., Bowden, T. J., Lomax, J. & Ellis, A. E. (1997). Antibody response and protection of Atlantic salmon (*Salmo salar*) immunised with an extracellular polysaccharide of *Aeromonas salmonicida*. *Fish & Shellfish Immunology* **7**, 1-16.

Brock, J. (1995). Lactoferrin: a multifunctional immunoregulatory protein. *Immunology Today* **16**, 417-419.

Brodsky, W. Y. & Uryvaeva, I. V. (1978). Cell polyploidy: its relation to tissue growth and function. *International Reviews of Cytology* **50**, 275-332.

Bruno, D. B. & Johnstone, R. (1990). Susceptibility of diploid and triploid Atlantic salmon to *Renibacterium salmoninarum. Bulletin of the European Association of Fish Pathologists* **10**, 45-47.

Brydges, K. & Benfey, T. J. (1991). Triploid brown trout (*Salmo trutta*) produced by hydrostatic pressure shock. *Bulletin of the Aquaculture Association of Canada* **91**, 31-33.

Bullen, J. J. & Armstrong, J. A. (1979). The role of lactoferrin in the bactericidal function of PMN leucocytes. *Immunology* **36**, 781-791.

Bye, V. J. & Lincoln, R. F. (1986). Commercial methods for the control of sexual maturation in rainbow trout. *Aquaculture* **57**, 229-309.

Chart, H. & Trust, T. J. (1983). Acquisition of iron by *Aeromonas salmonicida. Journal of Bacteriology* **156**, 758-764.

Cherfas, N. B., Kozinsky, O., Rothbard, S. & Hulata, G. (1990). Induced diploid gynogenesis and triploidy in ornamental (koi) carp, *Cyprinus carpio* L. 1. Experiments on the timing of temperature shock. *Bamidgeh.* **42**, 3-9.

Chourrout, D. (1987). Genetic manipulations in fish: review of methods. In *Selection, hybridization and genetic engineering in aquaculture*, pp. 15. Edited by K. Tiews. Berlin: Heenemann Verlags.

Chourrout, D., Chevassus, B., Kreig, F., Happe, A., Burger, G. & Renard, P. (1986). Production of second generation triploid and tetraploid rainbow trout by mating tetraploid males and diploid females-potential of tetraploid fish. *Theoretical & Applied Genetics* **72**, 193-206.

Chung, S. & Secombes, C. J. (1988). Analysis of events occurring within teleost macrophages during the respiratory burst. *Comparative Biochemistry and Physiology* **89B**, 539-544.

Congleton, J. L. & Wagner, E. J. (1991). Acute phase hypoferraemic response to lipopolysaccharide in rainbow trout. *Comparative Biochemistry and Physiology.* **98a**, 195-200.

Cossarini-Dunier, M., Desvaux, R. S. & Dorson, M. (1986). Variability in humoral responses to DNP-KLH of rainbow trout (*Salmo gairdneri*). Comparison of antibody kinetics and immunoglobulin spectrotypes between normal trout and trout obtained by gynogenesis or self-fertilisation. *Developmental and Comparative Immunology* **10**, 207-217.

Crosa, J. H. (1984). The relationship of plasmid-mediated iron transport and bacterial virulence. *Annual Reviews of Microbiology* **38**, 69-89.

Cuchens, M. A. & Clem, L. W. (1977). Phylogeny of lymphocyte heterogeneity. II. Differential effects of temperature on fish T-like and B-like cells. *Cellular Immunology* **34**, 219-230.

Davidson, G. A., Ellis, A. E. & Secombes, C. J. (1993). Route of immunization influences the generation of antibody secreting cells in the gut of rainbow trout. *Developmental and Comparative Immunology* **17**, 373-376.

Davidson, G. A., Lin, S. H., Secombes, C. J. & Ellis, A. E. (1997). Detection of specific and 'constitutive' antibody secreting cells in the gills, head kidney and peripheral blood leucocytes of dab (*Limanda limanda*). *Veterinary Immunology and Immunopathology* **58**, 363-374.

de Kinkelin, P., Dorson, M. & Hattenberger-Baudouy, A. M. (1982). Interferon synthesis in trout and carp after viral infection. *Developmental and Comparative Immunology* **Suppl 2.**, 167-174.

Desvaux, F. X., Cossarini-Dunier, M., Chilmozcyk, S. & Charlemagne, J. (1987). Antibody diversity in trout obtained by gynogenesis or self-fertilisation. Comparative analysis of the heavy chain spectrotypes. *Developmental and Comparative Immunology* **11**, 577-584.

Dorson, M., Chevassus, B. & Torhy, C. (1991). Comparative susceptibility of three species of char and of rainbow trout x char triploid hybrids to several pathogenic salmonid viruses. *Diseases of Aquatic Organisms* **11**, 217-224.

Dorson, M., De Kinkelin, P. & Torchy, C. (1992). Interferon synthesis in rainbow trout fry following infection with infectious pancreatic necrosis virus. *Fish & Shellfish Immunology* **2**, 311-313.

Douvas, G. S., May, M. H. & Crowie, A. J. (1993). Transferrin, iron and serum lipids enhance or inhibit *Mycobacterium avium* replication in human macrophages. *Journal of Infectious Diseases* **167**, 857-864.

Du Pasquier, L. (1982). Antibody diversity in lower vertebrates-why is it so restricted? *Nature* **296**, 311-313.

Elliot, J. W., Fowler, L. G. & Burrows, R. E. (1966). Effects of age, growth and diet on characteristics of salmon fingerlings. : Bureau of Sports Fisheries & Wildlife, U. S. Dept. of the Interior.

Ellis, A. E. (1981). Non-specific defence mechanisms in fish and their role in disease processes. In *International Symposium on Fish Biologics: Serodiagnostics & Vaccines*, pp. 337-352. Edited by S. Karger. Leetown, W. Va., USA: Basel.

Ellis, A. E. (1986). The function of teleost fish lymphocytes in relation to inflammation. *International Journal of Tissue Reactions* **8**, 263-270.

Ellis, A. E. (1987). Inhibition of *Aeromonas salmonicida* extracellular protease by α_2 macroglobulin in the serum of rainbow trout. *Microbial Pathogenesis* **3**, 167-177.

Ellis, A. E. (1990). Lysozyme Assays. In: *Techniques in Fish Immunology.* Eds. Stolen, J. S., Fletcher, T. C., Anderson, D. P., Kaattari, S. L., Rowley, A. F., Fairhaven: SOS Publications. p101-104.

Ellis, A. E., do Vale, A., Bowden, T. J., Thompson, K. & Hastings, T. S. (1997). *In vivo* production of A-protein, lipopolysaccharide, iron-regulated outer membrane proteins and 70-kDa serine protease by *Aeromonas salmonicida* subsp. *salmonicida*. *Fems Microbiology Letters* **149**, 157-163.

Ellis, A. E., Hastings, T. S. & Munro, A. L. S. (1981). The role of *Aeromonas salmonicida* extracellular products in the pathology of furunculosis. *Journal of Fish Diseases* **4**, 41-51.

Ellsaesser, C. F. & Clem, L. W. (1986). Haematological and immunological changes in channel catfish stressed by handling and transport. *Journal of Fish Biology* **28**, 511-521.

Evans, D. L. & Jaso-Friedmann, L. (1992). Non-specific, cytotoxic cells as effectors of immunity in fish. *Annual Review of Fish Diseases* **2**, 109-121.

Fankhauser, G., Crotta, R. & Perrot, M. (1942). Spontaneous and cold induced triploidy in the Japanese newt, *Triturus pyrrhogaster*. *Journal of Experimental Zoology* **89**, 167-181.

Fevolden, S. E., Roed, K. H. & Gjerde, B. (1994). Genetic components of post-stress cortisol and lysozyme activity in Atlantic salmon; correlations to disease resistance. *Fish & Shellfish Immunology* **4**, 507-519.

Finn, J. P. & Nielson, N. O. (1971). The inflammatory response of rainbow trout. *Journal of Fish Biology* **3**, 463.

Flajshans, M., Kvasnicka, P. & Rab, P. (1993). Genetic studies in tench (*Tinca tinca* L.): High incidence of spontaneous triploidy. *Aquaculture* **110**, 3-4.

Flajshans, M., Linhart, O. & Kvasnicka, P. (1993). Genetic studies of tench (*Tinca tinca* L.): Induced triploidy and tetraploidy and first performance data. *Aquaculture* **113**, 301-312.

Fletcher, T. C. & White, A. (1973). Lysozyme activity in the plaice (*Pleuronectes platessa* L.). *Experientia* **29**, 1283-1285.

Fletcher, T. C. & White, A. (1976). The lysozyme of the plaice (*Pleuronectes platessa* L.). *Comparative Biochemistry and Physiology* **558**, 207-210.

Freedman, S. J. (1991). The role of α_2 macroglobulin in furunculosis: a comparison of rainbow trout and brook trout. *Comparative Biochemistry and Physiology.* **98b**, 549-553.

Fyfe, L., Coleman, L. & Munro, A. L. S. (1988). The combined effect of isolated *Aeromonas salmonicida* protease and hemolysin on Atlantic salmon, *Salmo salar* L., compared with that of total extracellular product preparations. *Journal of Fish Diseases* **11**, 101-104.

Fyfe, L., Finley, A., Coleman, G. & Munro, A. L. S. (1986). A study of the pathological effect of isolated *Aeromonas salmonicida* extracellular protease on Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* **9**, 403-409.

Gjedrem, T. & Gjoeen, H. M. (1995). Genetic variation in susceptibility of Atlantic salmon, *Salmo salar* L., to furunculosis, BKD and cold water vibriosis. *Aquaculture Research* **26**, 129-134.

Graham, M. S., Fletcher, G. L. & Benfey, T., J. (1985). Effect of triploidy on blood oxygen content of Atlantic salmon. *Aquaculture* **50**, 133-139.

Graham, S. & Secombes, C. J. (1990). Do fish lymphocytes secrete interferon-γ? *Journal of Fish Biology* **36**, 563-573.

Gray, A. K., Evans, M. A. & Thorgaard, G. H. (1993). Viability and development of diploid and triploid salmonid hybrids. *Aquaculture* **112**, 2-3.

Greenlee, A. R., Kersten, C. A. & Cloud, J. G. (1995). Effects of triploidy on rainbow trout myogenesis *in vitro*. *Journal of Fish Biology* **46**, 381-388.

Griffin, B. R. (1983). Opsonic effect of rainbow trout antibody on phagocytosis of *Yersinia ruckeri* by trout leucocytes. *Developmental and Comparative Immunology* **7**, 253-259.

Grinde, B. (1989). Lysozyme from rainbow trout, *Salmo gairdneri*, as an antibacterial agent against fish pathogens. *Journal of Fish Diseases* **12**, 95-104.

Grinde, B., Lie, O., Poppe, T. & Salte, R. (1988). Species and individual variation in lysozyme activity in fish of interest in aquaculture. *Aquaculture* **68**, 299-304.

Guxiong, C. (1993). Induction of triploid red sea bream, *Pagrosomus major*, using cold shock. *Aquaculture* **111**, 314.

Happe, A., Quillet, E. & Chevassus, B. (1988). Early life history of triploid rainbow trout (*Salmo gairdneri* Richardson). *Aquaculture* **71**, 1-2.

Hershberger, W. K. & Pratschner, G. A. (1981). Iron binding capacities of six transferrin phenotypes of Coho salmon and potential fish health applications. *Progressive Fish Culturist* **43**, 27-31.

Hirst, I. D. & Ellis, A. E. (1996). Utilization of transferrin and salmon serum as sources of iron by typical and atypical strains of *Aeromonas salmonicida*. *Microbiology* , 1543-1550.

Hirst, I. D., Hastings, T. S. & Ellis, A. E. (1991). Siderophore production by *Aeromonas salmonicida*. *Journal of General Microbiology* **137**, 1185-1192.

Hjelmeland, K., Christie, M. & Raa, J. (1983). Skin mucus protease from rainbow trout, *Salmo gairdneri*, and its biological significance. *Journal of Fish Biology* **23**, 13-22.

Hollebecq, M. G. & Michel, C. (1989). Evidence for a maternal effect in furunculosis resistance of rainbow trout. *4th Conference of the European Association of Fish Pathologists*, pp. 51. Sanitiago de Compostela.

Holloway, H. L., Shoemaker, C. A. & Ottinger, C. A. (1993). Serum lysozyme levels in paddlefish and walleye. *Journal of Aquatic Animal Health* **5**, 324-326.

Inada, Y., Yoshimura, K. & Taniguchi, N. (1990). Study on the resistance to vibriosis in induced triploid ayu *Plecoglossus altivelis*. *Nippon Suisan Gakkaishi.* **56**, 1587-1591.

Jarvi, T. (1989). Synergistic effect on mortality in Atlantic salmon, *Salmo salar*, smolt caused by osmotic stress and presence of predators. *Environmental Biology of Fishes* **26**, 149-152.

Johnson, O. W., Dickhoff, W. W. & Utter, F. M. (1986). Comparative growth and development of diploid and triploid coho salmon, *Oncorhynchus kisutch*. The Second International Symposium on Genetics in Aquaculture. *Gall, G.A.E.* **57**, 1-4.

Johnstone, R. (1992). Production and performance of triploid Atlantic salmon in Scotland. *Scottish Aquaculture Research Report.*

Johnstone, R. (1996). Experience with salmonid sex reversal and triploidisation technologies in the United Kingdom. *Bulletin of the Aquaculture Association Canada*, 96-2.

Johnstone, R., McLay, H. A. & Walsingham, M. V. (1991). Production and performance of triploid Atlantic salmon in Scotland. *Proceedings Of The Atlantic Canada Workshop On Methods For The Production Of Non Maturing Salmonids: February* , 15-36.

Joosten, D. H. M., Aviles-Trigueros, M., Sorgeloos, P. & Rombout, J. H. W. M. (1995). Oral vaccination of juvenile carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*) with bioencapsulated *Vibrio anguillarum* bacterin. *Fish and Shellfish Immunology* **5**, 289-299.

Kaattari, S. L. & Piganelli, J. D. (1996). The specific immune system: Humoral defence. In: *The Immune System of Fish* Vol. 15 Fish Physiology. Edited by Iwama, G., Nakanishi, T.

Kaattari, S. L. & Tripp, R. A. (1987). Cellular mechanisms of glucocorticoid immunosuppresion in salmon. *Journal of Fish Biology* **31**, 129-132.

Kaattari, S. L. (1984). Development of a piscine paradigm of immunological memory. *Fish and Shellfish Immunology* **4**, 447-457.

Kakuta, I. & Kurokura, H. (1995). Defensive effect of orally administered bovine lactoferrin against *Cryptocaryon irritans* infection in red sea bream. *Fish Pathology* **30**, 289-290.

Karczewski, J. M., Sharp, G. J. E. & Secombes, C. J. (1991). Susceptibility of strains of *Aeromonas salmonicida* to killing by cell-free generated superoxide anion. *Journal of Fish Disease* **14**, 367-373.

Kavumpurath, S. & Pandian, T. J. (1990). Induction of triploidy in the zebrafish, *Brachydanio rerio* (Hamilton). *Aquaculture and Fisheries Management* **21**, 299-306.

Kavumpurath, S. & Pandian, T. J. (1992). The development of all-male sterile triploid fighting fish (*Betta splendens* Regan) by integrating hormonal sex reversal of broodstock and chromosome-set manipulation. *Bamidgeh.* **44**, 111-119.

Kawahara, E., Oshima, S. & Nomura, S. (1990). Toxicity and immunogenecity of *Aeromonas salmonicida* extracellular products to salmonids. *Journal of Fish Diseases* **13**, 495-503.

Kitamura, S., Ogata, H. & Onozato, H. (1991). Triploid male masu salmon *Oncorhynchus masu* show normal courtship behaviour. *Nippon Suisan Gakkaishi.* **57**, 2157.

Klempner, M. S., Dinarello, C. A. & Gallin, J. I. (1978). Human leucocytic pyrogen induces release of specific granule contents from human neutrophils. *Journal of Clinical Investigations* **61**, 1330-1336.

Koteeswaran, R., Sheela, S. G. & Pandian, T. J. (1995). Testosterone biosynthesis in triploid sterile male tilapia. *Current Science* **69**, 545-547.

Koumans Van Diepen, J. C. E., Harmsen, E. G. M. & Rombout, J. H. W. M. (1994). Immunocytochemical analysis of mitogen responses of carp (*Cyprinus carpio*) peripheral blood leucoytes. *Veterinary Immunology and Immunopathology* **42**, 209-219.

Krueger, R. & Kohlmann, K. (1993). Histological comparison of liver and kidney tissue of diploid and triploid female rainbow trout (*Oncorhynchus mykiss*). From Discovery To Commercialization. *Carrillo, M.* , 236.

Kusuda, R., Salati, F., Hamaguchi, M. & Kawai, K. (1991). The effect of triploidy on phagocytosis, leucocyte migration, antibody and complement levels of ayu *Plecoglossus altivelius*. *Fish and Shellfish Immunology* **1**, 243-249.

Kvingedal, A. M., Rorvik, K. A. & Alestrom, P. (1993). Cloning and characterisation of Atlantic salmon serum transferrin cDNA. *Molecular Marine Biology and Biotechnology* **2**, 233-238.

Lamas, J. & Ellis, A. E. (1994). Atlantic salmon (*Salmo salar*) neutrophil responses to *Aeromonas salmonicida*. *Fish and Shellfish Immunology* **4**, 201-219.

Le Morvan-Rocher, C., Troutad, D. & Deschaux, P. (1995). Effects of temperature on carp leucocyte mitogen-induced proliferation and non-specific cytotoxic activity. *Developmental and Comparative Immunology* **19**, 87-95.

Lefkowitz, D. L., Lincoln, J. A., Lefkowitz, S. S., Bollen, A. & Moguilevsky, N. (1997). Enhancement of macrophage-mediated bactericidal activity by macrophage-mannose receptor-ligand interaction. *Immunological and Cellular Biology* **75**, 136-141.

Lie, Æ., Evensen, Æ., SÆrensen, A. & FrÆysadal, E. (1989). Study on lysozyme activity in some fish species. *Diseases of Aquatic Organisms* **6**, 1-5.

Lin, S. H., Ellis, A. E., Davidson, G. A. & Secombes, C. J. (1999). Migratory, respiratory burst and mitogenic responses of leucocytes isolated from the gills of rainbow trout (*Onchorynchus mykiss*). *Fish & Shellfish Immunology* In press.

Lincoln, J. A., Lefkowitz, D. L., Cain, T., Castro, A., Mills, K. C., Lefkowitz, S. S., Moguilevsky, N. & Bollen, A. (1995). Exogenous myeloperoxidase enhances bacterial phagocytosis and intracellular killing by macrophages. *Infection & Immunity* **63**, 3042-3047.

Lincoln, R. F. (1981). Sexual maturation in triploid male plaice (*Pleuronectes platessa*) and plaice x flounder (*Platichthys flesus*). *Journal of Fish Biology* **19**, 415-426.

Linhart, O. & Flajshans, M. (1995). Triploidization of European catfish, *Silurus glanis* L., by heat shock. *Aquaculture Research* **26**, 367-370.

Lou, Y. D. & Purdom, C. E. (1984). Polyploidy induced by hydrostatic pressure in rainbow trout. *Journal of Fish Biology* **25**, 345-351.

Magarinos, B., Romalde, J. L., Lemos, M. L., Barja, J. L. & Toranzo, A. E. (1994). Iron uptake by *Pasteurella piscicida* and its role in pathogenicity for fish. *Applied and Environmental Microbiology* **60**, 2990-2998.

Malison, J. A., Kayes, T. B., Held, J. A., Barry, T. P. & Amundson, C. H. (1993). Manipulation of ploidy in yellow perch (*Perca flavescens*) by heat shock, hydrostatic pressure shock, and spermatozoa inactivation. *Aquaculture* **110**, 3-4.

Manning, M. J. & Nakanishi, T. (1996). The specific immune system: Cellular defences. In: *The Immune System of Fish* Vol. 15 Fish Physiology. Edited by Iwama, G., Nakanishi, T.

Matsubara, K., Arai, K. & Suzuki, R. (1995). Survival potential and chromosomes of progeny of triploid and pentaploid females in the loach, *Misgurnus anguillicaudatus*. *Aquaculture* **131**, 1-2.

McKay, L. R., Ihssen, P. E. & McMillan, I. (1992). Growth and mortality of diploid and triploid tiger trout (*Salmo trutta* x *Salvelinus fontinalis*). *Aquaculture* **106**, 3-4.

McNab, A., Martin, J. & Landless, P. (1987). An environmental assessment of fish farms. : Countryside Commission for Scotland.

Miller, N. W. & Clem, L. W. (1984). Temperature-mediated processes in teleost immunity: differential effects of temperature on catfish in vitro antibody responses to thymus-dependent and thymus-independent antigens. *Journal of Immunology* **133**, 2356-2359.

Mock, A. & Peters, G. (1990). Lysozyme activity in rainbow trout, *Onchorynchus mykiss* (Walbaum), stressed by handling, transport and water pollution. *Journal of Fish Biology* **37**, 873-885.

Moeyner, K., Roeed, K. H., Sevatdal, S. & Heum, M. (1993). Changes in non-specific immune parameters in Atlantic salmon, *Salmo salar* L., induced by *Aeromonas salmonicida* infection. *Fish and Shellfish Immunology* **3**, 253-265.

Muona, M. & Soivio, A. (1992). Changes in plasma lysozyme and blood leucocyte levels of hatchery-reared Atlantic salmon (*Salmo salar* L.) and sea trout (*Salmo trutta* L.) during parr-smolt transformation. *Aquaculture* **106**, 75-87.

Murray, C. K. & Fletcher, T. C. (1976). The immunohistochemical localisation of lysozyme in plaice (*Pleuronectes platessa* L.) tissues. *Journal of Fish Biology* **9**, 329-334.

Myers, J. M. & Hershberger, W. K. (1991). Early growth and survival of heat-shocked and tetraploid-derived triploid rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **96**, 97-107.

Nilsson, S. & Holmgren, S. (1992). Cardiovascular control by purines, 5-hydroxytryptamine and neuropeptides. In *Fish Physiology*, pp. 301-341. Edited by W. S. Hoar, D. J. Randall & A. P. Farrell. London: Academic Press.

Nottaga, A. S. & Birbeck, T. H. (1987). Production of proteinase during experimental infection of *Ostrea edulis* L. larvae with *Vibrio alginolyticus* NCMB 1339 and the antigenic relationship between proteinases produced by marine vibriosis pathogenic for fish and shellfish. *Journal of Fish Diseases* **10**, 265-273.

Ojolick, E. J., Cusack, R., Benfey, T. J. & Kerr, S. R. (1995). Survival and growth of all-female diploid and triploid rainbow trout (*Oncorhynchus mykiss*) reared at chronic high temperature. *Aquaculture* **131**, 3-4.

Otto, B. R., Vught, A. M. J. J. V. & Maclaren, D. M. (1992). Transferrins and heme compounds as iron sources for pathogenic bacteria. *Critical reviews in Microbiology* **18**, 217-233.

Ourth, D. D. (1980). Secretory IgM, lysozyme, and lymphocytes in the skin mucus of the channel catfish, *Ictalurus punctatus*. *Developmental and Comparative Immunology* **4**, 65-74.

Partula, S., de Guerra, A., Fellah, J. S. & Charlemagne, J. (1996). Structure and diversity of the T cell receptor α-chain in a teleost fish. *Journal of Immunology* **157**, 207-212.

Partula, S., Fellah, J. S., de Guerra, A. & Charlemagne, J. (1994). Identification of cDNA clones encoding the T-cell receptor β-chain in the rainbow trout, *Onchorynchus mykiss*. *Current Reviews of the Academy of Sciences, Paris, Life Sciences* **317**, 765-770.

Pepper, V. A. (1991). Proceedings of the Atlantic Canada Workshop on Methods for the Production of Non-maturing Salmonids: February 19-21, 1991, Dartmouth, Nova Scotia. *Canadian Technical Report on Fisheries and Aquatic Science* .

Persijn, P., Van der Slik, W. & Reithorst, A. (1971). Determination of serum iron and latent iron binding capacity. *Clinica et Chimica Acta.* **35**, 91-98.

Peters, G., Faisal, M., Lang, T. & Ahmed, I. (1988). Stress caused by social interaction and its effect on susceptibility to *Aeromonas hydrophilia* infection in rainbow trout *Salmo gairdneri. Diseases of Aquatic Organisms* **4**, 83-89.

Pickering, A. D. & Pottinger, T. G. (1987). Crowding causes prolonged leucopenia in salmonid fish, despite inter-renal acclimation. *Journal of Fish Biology* **32**, 701-712.

Pilstrom, L. & Bengten, E. (1996). Immunoglobulin in fish- genes, expression and structure. *Fish and Shellfish Immunology* **6**, 243-262.

Purdom, C. E. (1983). Genetic engineering by the manipulation of chromosomes. *Aquaculture* **33**, 287-300.

Quillet, E. & Gaignon, J. L. (1990). Thermal induction of gynogenesis and triploidy in Atlantic salmon (*Salmo salar*) and their potential interest for aquaculture. *Aquaculture* **89**, 3-4.

Ramsay, W. M. N. (1958). Plasma iron In: Advances in Clinical Chemistry Vol. 1 Edited by Sobotka, H. and Stewart, C. P. Academic Press.

Ravndal, J., Lovold, T., Bentsen, H. B., Roed, K. H., Gjedrem, T. & Rorvik, K. A. (1994). Serum iron levels in farmed Atlantic salmon: family variation and associations with disease resistance. *Aquaculture* **125**, 37-45.

Recoubratsky, A. V., Gomelsky, B. I., Emelyanova, O. V. & Pankratyeva, E. V. (1992). Triploid common carp produced by heat shock with industrial fish-farm technology. *Aquaculture* **108**, 1-2.

Reité, O. B. (1997). Mast cells/eosinophilic granule cells of salmonids: staining properties and responses to noxious agents. *Fish and Shellfish Immunology* **7**, 567-584.

Reite, O. B. (1998). Mast cells/ eosinophilic granule cells of teleostean fish: a review focusing on staining properties and functional responses. *Fish and Shellfish Immunology* **8**, 489-513.

Roed, K. H., Dehli, A. K., Flengsrud, R., Midthjell, L. & Rorvik, K. A. (1995). Immunoassay and partial characterisation of serum transferrin from Atlantic salmon (*Salmo salar* L.). *Fish and Shellfish Immunology* **5**, 71-80.

Roeed, K. H., Fjalestad, K., Larsen, H. J. & Midthjel, L. (1992). Genetic variation in hemolytic activity in Atlantic salmon (*Salmo salar* L.). *Journal of Fish Biology* **40**, 739-750.

Roitt, I., Brostoff, J. & Male, D. (1993). *Immunology*, 3rd edn: Mosby.

Sadler, J., Wells, R. M. G., Pankhurst, P. M. & Pankhurst, N. W. (1998). Blood oxygen transport, rheology and haematological responses to confinement stress in diploid and triploid Atlantic salmon, *Salmo salar*. In Press .

Sakai, D. K. (1984). Opsonisation by fish antibody and complement in the immune phagocytosis by peritoneal exudate cells isolated from salmonid fishes. *Journal of Fish Diseases* **7**, 29-38.

Sakai, D. K. (1992). Repertoire of complement in immunological defence mechanisms of fish. *Annual Review of Fish Diseases* ,**2** 223-247.

Sakai, M., Kobayashi, M. & Yoshida, T. (1995). Activation of rainbow trout, *Onchorynchus mykiss*, phagocytic cells by administration of bovine lactoferrin. *Comparative Biochemistry and Physiology* **110b**, 755-759.

Sakai, M., Otubo, T., Atsuta, S. & Koybayashi, M. (1993). Enhancement of resistance to bacterial infection in rainbow trout, *Onchorynchus mykiss*, by oral administration of bovine lactoferrin. *Journal of Fish Diseases* **16**, 239-247.

Salte, R., Norberg, K., Odegaard, O. R., Arnesen, J. A. & Olli, J. J. (1993). Exotoxin-induced consumptive coagulopathyin Atlantic salmon *Salmo salar* L.: inhibitory effects of exogenous antithrombin and α_2 macroglobulin on *Aeromonas salmonicida* serine protease. *Journal of Fish Diseases* **16**, 425-435.

Sattaur, O. (1989). The threat of the well-bred salmon. *New Scientist* , 54-60.

Schreck, C. B. (1982). Stress and rearing of salmonids. *Aquaculture* **28**, 241-249.

Schreck, C. B. (1996). Immunomodulation: Endogenous factors. In: *The Immune System of Fish* Vol. 15 Fish Physiology. Edited by Iwama, G., Nakanishi, T.

Secombes, C. J. & Fletcher, T. C. (1992). The role of phagocytes in the protective mechanisms of fish. *Annual Reviews of Fish Diseases* ,**2** 53-71.

Secombes, C. J. (1985). The *in vitro* formation of teleost multinucleate giant cells. *Journal of Fish Diseases* **8**, 461-464.

Secombes, C. J. (1990). Isolation of salmonid macrophages and analysis of their killing activity. In*: Techniques in Fish Immunology.* Eds. Stolen, J. S., Fletcher, T. C., Anderson, D. P., Kaattari, S. L., Rowley, A. F., Fairhaven: SOS Publications. p137-154.

Secombes, C. J. (1996). The non-specific immune system:Cellular defences. In *Fish Physiology*, pp. 43. Edited by G. Iwama & T. Nakanishi.

Secombes, C. J., Hardie, L. J. & Daniels, G. (1996). Cytokines in fish: An update. *Fish and Shellfish Immunology* **6**, 291-304.

Sezaki, K., Watabe, S., Tsukamoto, K. & Hashimoto, K. (1991). Effects of increase in ploidy status on respiratory function of ginbuna, *Carassius auratus* langsdorfi (Cyprinidae). *Comparative Biochemistry and Physiology, A.* , 1-2.

Sharp, G. J. E. & Secombes, C. J. (1992). Observations on the killing *of Aeromonas salmonicida* by rainbow trout (*Oncorhynchus mykiss* (Walbaum)) macrophages. .

Sharp, G. J. E. & Secombes, C. J. (1993). The role of reactive oxygen species in the killing of the bacterial fish pathogen *Aeromonas salmonicida* by rainbow trout macrophages. *Fish and Shellfish Immunology* **3**, 119-129.

Small, S. A. & Benfey, T. J. (1987). Cell size in triploid salmon. *Journal Experimental Zoology* **241**, 339-342.

Small, S. A. & Randall, D. J. (1989). Effects of triploidy on the swimming performance of coho salmon (*Oncorhynchus kisutch*). *Canadian Journal of Fisheries and Aquatic Science* **46**, 243-245.

Suzuki, Y. & Iida, T. (1992). Fish granulocytes in the process of inflammation. *Annual Reviews of Fish Diseases* **2**, 149-160.

Swarup, H. (1959). Production of triploidy in *Gasterosteus aculeatus. Journal of Genetics* **56**, 129-142.

Thorgaard, G. H. & Allen, S. K., Jr. (1987). Chromosome manipulation and markers in fishery management. In: *Population Genetics and Fishery Management.* Edited by Ryman, N. , 319-331.

Thorgaard, G. H. & Gall, G. A. E. (1979). Adult triploids in a rainbow trout family. *Genetics* **93**, 961-973.

Thorgaard, G. H. (1986). Ploidy manipulation. *Aquaculture* **57**, 57-64.

Toney, M. L. & Coble, D. W. (1980). Mortality, haematocrit, osmolality, electrolyte regulation and fat depletion of young-of-the-year freshwater fishes under simulated winter conditions. *Canadian Journal of Fisheries and Aquatic Sciences* **37**, 225-232.

Travis, J. & Salvesen, G. S. (1983). Human plasma proteinase inhibitors. *Annual Review of Biochemistry* **52**, 655-709.

Travis, J., Potempa, J. & Maeda, H. (1995). Are bacterial proteinases pathogenic factors? *Trends in Microbiology* **3**, 405-407.

Utter, F. M., Ames, W. E. & Hodgins, H. O. (1970). Transferrin polymorphisms in coho salmon. *Journal of the Fisheries Research Board of Canada* **27**, 2371-2373.

Utter, F. M., Johnson, O. W., Thorgaard, G. H. & Rabinovitch, P. S. (1983). Measurement and potential applications of induced triploidy in Pacific salmon. *Aquaculture* **35**, 125-135.

Vallejo, A. N., Miller, N. W. & Clem, L. W. (1992). Antigen processing and presentation in teleost immune responses. *Annual Reviews of Fish Diseases* **2**, 73-89.

Virtanen, E., Forsman, L. & Sundby, A. (1990). Triploidy decreases the aerobic swimming capacity of rainbow trout (*Salmo gairdneri*). *Comparative Biochemistry and Physiology, A.* , 117-121.

Warr, G. W. (1995). The immunoglobulin genes in fish. *Developmental and Comparative Immunology* **19**, 1-12.

Warr, G. W. (1997). The adaptive immune system of fish. In *Fish Vaccinology*, pp. 15-21. Edited by R. Gudding, A. Lillehaug, P. J. Midtlyng & F. Brown. Oslo: Developments in Biological Standardization.

Wedemeyer, G., Ross, A. J. & Smith, L. (1968). Some metabolic effects of bacterial endotoxins in salmonid fishes. *Journal of Fisheries Research Board of Canada* **26**, 115-122.

Weinberg, E. D. (1984). Iron withholding: a defence against intracellular infection and neoplasia. *Physiology Reviews* **64**, 65-102.

Weinberg, E. D. (1989). Cellular regulation of iron assimilation. *The Quarterly Review of Biology* **64**, 261-290.

Weinberg, E. D. (1992). Iron depletion: A defence against intracellular infection and neoplasia. *Life Sciences* **50**, 1289-1297.

Whaley, K. & Lemercier, C. (1993). The Complement System In: *The Natural Immune System: Humoral Factors.* Edited by E. Sim. IRL Press.

Wiegertjes, G. F., Stet, R. J. M. & van Muiswinkel, W. B. (1995). Investigations into the ubiquitous nature of high and low immune responsiveness after divergent selection for antibody production in common carp (*Cyprinous carpio* L.). *Veterinary Immunology and Immunopathology* **48**, 355-366.

Wilson, M. R., Zhou, H., Bengten, E., Clem, L. W., Stuge, T. S., Warr, G. W. & Miller, N. W. (1999). T cell receptors in channel catfish: structure and expression of TCR α and β genes. *Molecular Immunology* in press.

Xinqi, Y., Minrong, C., Xiaomu, Y. & Hongxi, C. (1993). Biological characters and growth rates of diploid and triploid Japanese phytophagous crucian carp. *Aquaculture* **111**, 320.

Yamamoto, A. & Iida, T. (1994). Haematological characteristics of triploid rainbow trout. *Fish Pathology* **29**, 239-243.

Yamamoto, A. & Iida, T. (1995). Bactericidal activity of serum of all-female triploid rainbow trout. *Fish Pathology* **30**, 123-124.

Yamamoto, A. & Iida, T. (1995). Non-specific defence activities of triploid rainbow trout. *Fish Pathology* **30**, 107-110.

Yamamoto, A. & Iida, T. (1995). Susceptibility of triploid rainbow trout to IHN, furunculosis and vibriosis. *Fish Pathology* **30**, 69-70.

Yano, T. (1992). Assays of hemolytic complement activty. In*: Techniques in Fish Immunology.* Eds. Stolen, J. S., Fletcher, T. C., Anderson, D. P., Kaattari, S. L., Rowley, A. F., Fairhaven: SOS Publications. p101-104.

Yano, T. (1996). The non-specific immune system: Humoral defence. In *The Immune System of Fish.* Vol. 15 Fish Physiology Edited by G. Iwama & T. Nakanishi

Yin, Z., Lam, T. J. & Sin, Y. M. (1995). The effects of crowding stress on the non-specific immune response in fancy carp (*Cyprinous carpio* L.). *Fish and Shellfish Immunology* **5**, 519-529.

Yousif, A. N., Albright, L. J. & Evelyn, T. P. T. (1994). *In vitro* evidence for the antibacterial role of lysozyme in salmonid eggs. *Diseases of Aquatic Organisms* **19**, 15-19.