Studies on Resistance to Infectious Pancreatic Necrosis Virus in Atlantic salmon (*Salmo salar*)

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Declaration

I declare that this thesis has been compiled by myself and is the result of my own investigations. It has not been submitted for any other degree and all sources of information have been duly acknowledged.

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Abstract

Infectious pancreatic necrosis virus (IPNV) is responsible for serious mortalities in post-smolt Atlantic salmon (*Salmo salar*) annually in both Scotland and Norway. The huge financial losses involved and the lack of control measures have prompted attempts to produce IPN vaccines and disease resistant strains of fish. A characteristic of IPNV is its ability to persist in a latent carrier state and to ensure stocks of fish are truly virus free there is a need for highly sensitive diagnostic methods.

In this study microsatellites were employed to assign fish susceptible to IPN in the field to families. Laboratory challenges were performed on 24 families and various humoral immune parameters were measured. A variety of diagnostic techniques were also developed, their sensitivity and specificity evaluated and compared to tissue culture.

Parental Atlantic salmon and offspring susceptible to IPN in the field were genotyped using a published multiplex microsatellite PCR adapted for use with fluorescent genotyping systems. The reliability and utility of the genotyping system was evaluated with positive results. The majority of susceptible fish could not be assigned to parents since the fish were found to be from a mixed stock.

Various experimental challenges with IPNV infected tissue homogenate were performed on post-smolts, however they did not produce mortality or induce clinical IPN. As a result of this virus titre was used as an indirect measurement of disease resistance. Atlantic salmon post-smolts were found to effectively clear cell cultured IPNV, isolate Br from Shetland. A second Shetland isolate (Os) was found to actively replicate in the fish while isolate Br was rapidly cleared. To increase virulence isolate

Os was passaged once further through fish prior to use in challenging 24 families of PIT tagged Atlantic salmon. At week 3 mean viral titres for each family ranged from $10^{0.8}$ to 10^{11} $TCID_{50}$/g kidney. Using these data six families were targeted to follow through to week 7, two with low titres, two with medium titres and two with high titres of IPNV. At week 7 virus titres had decreased to between $10^{0.8}$ and $10^{3.5}$ $TCID_{50}$/g kidney, many of the fish in the families with low IPNV titres at week 3 had cleared the virus. Lysozyme and neutralising antibody responses were measured, however little or no correlation was found between either parameter and IPNV titre at weeks 3 or 7. The control fish were found to be positive for IPNV despite earlier negative testing. Reasons for this were unclear, the fish may have been exposed to the virus through the seawater inflow or alternatively the fish may have been carriers of the virus.

Diagnostic techniques employed to detect IPNV were RT-PCR, ELISA and immunohistochemistry; cell culture was used as a comparison to the RT-PCR and ELISA. The RT-PCR was developed using two published IPNV primer sets both of which amplified regions on VP2, they were found to be specific to IPNV and together amplified 9 out of 10 serotypes of IPNV. The most sensitive primer set detected between 10^{7} and 10^{8} $TCID_{50}$ IPNV/ml in spiked tissue, in comparison to the ELISA which detected $10^{12.5}$ $TCID_{50}$ IPNV/ml in tissue homogenate. The AS-1 monoclonal antibody was used successfully in immunohistochemistry to identify IPNV in fixed tissue. The antibody was found to identify IPNV in pancreatic lesions from infected fish, but did not detect the virus in the gut. All of these methods were found to be less sensitive than cell culture, however they are very rapid and ELISA and RT-PCR may be used to confirm IPNV infection once CPE has been visualised in cell culture.

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Abbreviations

µl	microlitre
µM	micromolar
ANOVA	analysis of variance
APS	ammonium persulphate
AS	Atlantic salmon cells
BF-2	bluegill fibroblast cells
Br	Brindister IPNV isolate
BSA	bovine serum albumin
BSNV	Blotched Snakehead Virus
CEFAS	Centre for the Environment Fisheries and Aquaculture
CHSE-214	Chinook salmon embryo cells
CPE	cytopathic effect
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsRNA	double stranded RNA
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagles minimum essential medium
FBS	foetal bovine serum
fg	fantogram
FSG	fish skin gelatin
H & E	haematoxylin and eosin
HBSS	Hank's balance salt solution
HEWL	hen egg white lysozyme
HRP	horseradish peroxidase
HW	Hardy-Weinberg
IBDV	Infectious bursal disease virus
IFAT	immunofluorescence test
IFN	interferon
IgG	Immunoglobulin G
IHC	immunohistochemistry

IHNV	Infectious Haematopoeitic Necrosis Virus
IP	intra-peritoneal
IPNV	infectious pancreatic necrosis virus
ISAV	Infectious Salmon Anaemia Virus
M	Molar
mg	milligram
ml	millilitre
mM	millimolar
ng	nanogram
Os	Out Skerries IPNV isolate
PBS	phosphate buffered saline
PBSN	PBS thiomersol with 1 % sodium azide
PBST	PBS-Tween20
PCR	polymerase chain reaction
PCV	packed cell volume
pg	picogram
PIT tags	passive integrated transponders
PNNV	Piscine Nervous Necrosis Virus (Nodavirus)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RTG-2	rainbow trout gonad cells
RT-PCR	reverse transcription PCR
S½	salmon advanced to go to sea within 6 months
SAPU	Scottish Antibody Production Unit
SERAD	Scottish Executive Rural Affairs Department
SHK	Salmon Head Kidney cells
SPB	sodium phosphate buffer
SSN-1	Striped Snakehead fry/peduncle cells
ssRNA	single stranded RNA
TBE	tris-borate EDTA buffer
TBS	tris buffered saline
TCID	tissue culture infective dose
TE	tris-EDTA buffer

TMB	3,3', 5,5', tetramehyl-benzidine dihydrochloride
TNE	tris-NaCl-EDTA buffer
TSA	tryptic-soya agar
UV	ultra-violet
VNTR	variable number tandem repeats

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1. General Introduction

1.1. Introduction

In an intensive culture system such as the farming of Atlantic salmon (*Salmo salar*) fish are heavily stocked and fed artificial diets that enable the farmer to yield far higher productivity than at natural levels. Fish maintained in such systems are under many pressures; for example, crowding, handling, confinement and fluctuating water conditions. These conditions can be particularly stressful and may mean the fish are more vulnerable to the threat of disease. Fish susceptibility to disease depends on age, genetic strain differences, non-specific immune capabilities and immunocompetence when in the presence of a pathogen (Shepherd, 1992).

There are many pathogens that threaten the health of farmed fish. In the case of bacterial diseases antibiotics can be used to treat outbreaks, and indeed with many bacterial pathogens vaccines have been developed. Another alternative after a disease outbreak is to cull all remaining fish, disinfect the holding facilities and re-stock with disease free fish, however this method is not always practical. Viral diseases can be particularly problematic due to a lack of adequate control measures. There are no effective drug treatments for viral infections and for many fish viruses vaccination has not yet been possible. Infectious pancreatic necrosis virus (IPNV) is the causal agent of infectious pancreatic necrosis (IPN), an economically important disease of salmonid fish (Wolf, 1988). Although vaccination against IPNV may eventually be a possibility there are currently no control measures available. One possibility to reduce the impact of IPNV is to improve resistance to the virus by selection.

1.2. Infectious Pancreatic Necrosis

1.2.1. History

IPN was initially recognised as a problem in brook trout (*Salvelinus fontinalis*) farming in North America in the early part of this century and was originally termed acute catarrhal enteritis (M'Gonigle, 1940). The causal agent was then unknown. The disease was initially thought to be caused by a physiological problem at first feeding and the choice of food considered to be to blame. The pathology of the disease was first described by Davis in 1953, but it was not until 1955 that Wood, Snieszko and Yasutake noted that a disease with the same features as enteritis was highly contagious and upon histopathological examination termed the disease infectious pancreatic necrosis. The causative agent was isolated in 1957 from ground fish tissue; it was not until 1960 that the viral etiology was established (Wolf, Snieszko, Dunbar and Pyle, 1960, Wolf, Dunbar and Snieszko, 1960). Since the isolation of IPNV in the USA and Canada it has been shown to have a wide geographical distribution (Hudson, Bucke and Forrest, 1981). The first isolation in Europe was in France in 1965 (Besse and de Kinkelin, 1965), very shortly afterwards the virus was found in Denmark (Vestergard Jorgensen and Bregnballe, 1969), Sweden (Ljunberg and Vestergard Jorgensen, 1973), Italy (Ghittino, 1972), and Japan (Sano, 1971). IPNV was first isolated in the UK in rainbow trout fry (*Oncorhynchus mykiss*) in Scotland (Ball, Munro, Ellis, Elson, Hodgkiss and McFarlane, 1971). Much later IPNV was isolated in Chile (McAllister and Reyes, 1984).

Clinical IPN had traditionally been observed in brook trout and rainbow trout less than six months of age (Christie, 1997). IPNV was first isolated from Atlantic salmon fry in Canada (MacKelvie and Artsob, 1969), since this time the incidence of IPNV in

Atlantic salmon has increased, particularly in Scottish fry between 1979 and 1981 (Smail and Munro, 1985). Atlantic salmon post-smolts infected with IPNV was first reported in Scotland (Smail and Munro, 1985). Since this time the occurrence of IPN in Atlantic salmon farming has increased and is now a problem for post-smolts in their first year in seawater both in Scotland and Norway. In Norway the incidence of clinical IPN in Atlantic salmon farms was 39% in 1991 and had increased to 61% in 1995 (Christie, 1997). IPN is thought to cost Norwegian fish farmers approximately £40 million per year, this includes mortalities (worth about £16 million) and also reduced growth in surviving fish (Cripps, 1999).

1.2.2. The virus

IPNV is classified as a birnavirus and is a medium sized, unenveloped bi-segmented double stranded (ds) RNA containing virus (Dobos, 1995). Examination by electron microscopy of purified virus revealed single shelled icosahedrons having a diameter of 60 nm (Dobos, Hallett, Kells, Sorenson and Rowe, 1977). The viral capsid consists of three proteins, VP1, VP2 and VP3 (Dorson, 1988). VP1 is an internal protein that surrounds both segments of dsRNA, VPg is the genome linked form of VP1 (Bernard and Bremont, 1995). VP2 is the major outer capsid protein and makes up 62% of the virion protein (Dobos *et al.*, 1977). VP3 consists of two molecules of very similar molecular weight, the smaller one (VP3a or VP4) is thought to be the cleavage product of the other (Dobos and Rowe, 1977). VP3 was considered to be an internal protein (Dobos *et al.*, 1977), however recent work has shown that purified virus reacts with anti-VP3, therefore part of this protein may be expressed on the virion surface (Nicholson, 1993, Park and Jeong, 1996). Genome segment A (the larger RNA

segment) encodes for a polyprotein which generates the major capsid proteins VP2 and VP3, genome segment B encodes for VP1 (Dobos, 1995).

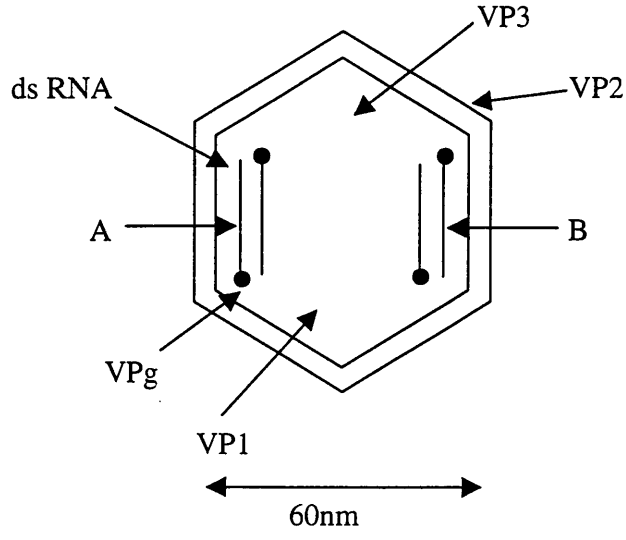


Figure 1.1: IPNV virion (Dobos, 1995)

Sano, Okamoto, Fukuda, Saneyoshi and Sano (1992) concluded that RNA segment A was responsible for the fatal disease in rainbow trout fry. Genetic virulence was determined by reassorting segments A and B between IPNV-Buhl strain and eel virus European (EVE). EVE was avirulent to rainbow trout prior to reassortment with segment A from IPNV-Buhl, with the replacement EVE became virulent to rainbow trout fry.

IPNV replicates in a variety of teleost fish cell lines. Malsberger and Cerini (1963) showed that the virus replicates in the cytoplasm and a single cycle of replication takes 16-20 hours at 22°C resulting in characteristic cytopathic effect (CPE). Binding experiments have shown that it takes 2-3 hours at 4°C for the virus to saturate all

binding sites, and that VP2 is the cell attachment protein of the virion (Marshall and Dobos, unpublished, cited in Dobos, 1995).

Studies have shown IPNV serotype variation between broad geographical areas (Melby, Caswell-Reno and Falk, 1994). The different serotypes have been grouped into two groups based on their cross reactivity, European serotypes such as Sp, Ab, He and Te, the West Buxton serotype (VR299) from the USA and Canadian serotypes Ja, C1, C2, and C3 make up serogroup A (Hill and Way, 1995). While serogroup B consists of only one serotype to date, TV-1. From the European serotypes Ab has been shown to display a low virulence while Sp is often highly virulent (Wolf, 1988). There has been a suggestion that IPNV isolates from Shetland may be a new serotype based on the fact that IPNV in Shetland appears to be very virulent (Ross and Munro, 1995). However this was proven unlikely to be the case. Pryde, Melvin and Munro (1993) found that upon sequencing the VP2 protein of Sp. (A2) and the Shetland isolate they differed by less than 3%, indicating that the Shetland isolate is in fact A2.

1.2.3. Disease signs

Clinical signs of IPN disease include darkened pigmentation, a pronounced distended abdomen and a corkscrewing and spiralling swimming motion in severely moribund fish (Hill, 1982). Exopthalmia is another common sign along with pale gills and long white faecal casts (Wolf, 1988). Internal signs include a build up of ascitic fluid in the body cavity, the stomach and anterior intestine contain a pathognomonic clear to milky cohesive mucus (Wolf, 1988). The infection produces pancreatic necrosis, with the lesions characterised by extensive degeneration of acinar cells and marked infiltration of mononuclear cells (Swanson and Gillespie, 1979). Zymogen granules may be released

and fatty tissue may also show signs of necrosis (Wolf 1988, Smail, Bruno, Dear, McFarlane and Ross, 1992). Swanson and Gillespie (1979) and Taksdal, Strangeland and Danniveg (1997) showed degeneration of liver parenchymal cells, characterised by pyknosis and karyolysis of hepatocytes. Smail, McFarlane, Bruno and McVicar (1995) found a marked feature of pathology in Atlantic salmon was prominent gut enteritis with sloughing of the mucosa to form a catarrhal exudate. This was thought to disturb digestive function by leading to malabsorption of the gut contents and an interruption of the normal passage of food through the gut. They concluded that the effects on gut physiology caused by IPNV might be more acute than the effects of pancreatic necrosis.

1.2.4. Transmission

Various studies have suggested that IPNV may be transmitted vertically. Evidence for vertical transmission has been demonstrated by Bullock, Rucker, Amend, Wolf and Stuckey (1976) who showed that brook trout fry suffered IPN mortalities despite disinfection of eggs from infected brood stock with iodine. Dorson and Torchy (1985) obtained transmission via infected sperm, suggesting that IPN is adsorbed to the spermatozoa and carried inside the egg. It was thought unlikely that an infected embryo would survive and develop; however the virus may persist in the chorion or in the perivitelline space. Ahne and Negele, (1985) concluded that hardened egg shells can take up IPNV from the water because of their lobed and porous nature, the pores were thought to provide anchorage for the virus possibly protecting it from treatment and water flow. It is known that IPNV is shed with faeces and reproductive fluids (Frantsi and Savan, 1971) and it is possible that this may contaminate surrounding areas and organisms leading to horizontal transmission.

1.2.5. Carrier status

A particular feature of IPNV infection is that many fish become virus carriers for long periods, possibly for life, indicating the chronic and persistent nature of the infection (Smail and Munro, 1985). IPNV carriers are asymptomatic but persistently infected fish and may carry the virus in many organs (Bootland, Dobos and Stevenson, 1991). MacDonald and Yamamoto (1977) demonstrated that IPNV exhibited an interference phenomenon that resulted in the survival of infected cell cultures, the responsible factor was found to co-purify with infectious virus suggesting the involvement of a defective interfering virus particle. It is thought that defective interfering particles (DI) are the result of mistakes during viral replication, DI are dependent on infectious virus to replicate but also interfere with the replication of infectious virus. Isolation in cell culture is likely to be affected by defective interfering particles and carrier status may be related to their presence (MacDonald and Yamamoto, 1977).

Although it has been shown that carrier fish do mount a humoral immune response they still periodically shed infectious virus in their faeces and reproductive fluids (Hill, 1982). This periodical shedding indicates that carrier parents may pass the virus onto their progeny. In 1992 Munro and Smail published the results of a ten year (1982-1991) monitoring programme for IPNV in both freshwater and seawater Atlantic salmon farm sites in Scotland. The annual sampling of broodstock reproductive fluids appeared to have reduced the presence of the virus in freshwater, however the incidence of IPNV in saltwater had increased. It was thought possible that if the virus was present in fish in freshwater and escaping detection there may be a mechanism as yet not described for an RNA virus to survive at levels below existing detection methods up to the smolt stage. IPNV has been commonly isolated from the kidney and use of this organ has been

known to detect carrier status, suggesting that leucocytes may harbour the virus (Knott and Munro, 1986, Johansen and Sommer, 1995). Johansen and Sommer (1995) concluded that non-cytolytic infected cells may protect the virus from the hosts' immune response leading to a carrier condition.

1.2.6. Diagnosis

The standard method for the detection of aquatic birnaviruses requires inoculation of fish cell cultures with homogenates of fish tissue (Amos, 1985). Once cytopathic effect (CPE) has been detected the virus must be further identified by titration neutralisation. These methods are time consuming and relatively slow. A sensitive, rapid diagnostic method is needed to detect IPNV (Dixon and Hill, 1983, Davis, Laidler, Perry, Rossington, Alcock, 1994, Lopez-Lastra, Gonzalez, Jashes, Sandino, 1994), as there is a lack of adequate disease control measures. There are various methods that can be used to detect and identify IPNV, such as ELISA (Dixon and Hill, 1983), immuno dot blot (Babin, Hernandez, Sanchez, Dominquez, 1991), cDNA probes (Dopazo, Hetrick, Samal, 1994), *in-situ* hybridisation and immunohistochemistry (Biering and Bergh, 1996). However, many are not as sensitive as cell culture, indeed ELISA although rapid only detects IPNV in clinical cases (Rodak, Pospisil, Tomanek, Vesely, Obr, Valicek, 1988). More recently research has turned to developing an RT-PCR (reverse transcription-polymerase chain reaction) to detect IPNV (Lopez-Lastra *et al.* 1994, Suzuki, Hosono, Kusuda, 1997, Wang, Wi, Lee, 1997). This method has proven to be very sensitive using purified virus, (Wang *et al.*, 1997) however its sensitivity using organ homogenates is not known.

1.2.7. Viral hosts

Although IPN is often thought of as a salmonid disease, many other species of fish have also been found to harbour IPNV, including eels (*Anguilla anguilla*) (Hudson, Bucke and Forrest, 1981), goldfish (*Carassius auratus* L.) and discus fish (*Symphysodon discus*) (Adair and Ferguson, 1981). The virus has also been isolated from marine shellfish, for example the flat oyster (*Ostrea edulis*), the edible mussel (*Mytilus edulis*), and the common periwinkle (*Littorina littorea*) (Hill and Way, 1995). It is possible however that the virus may not be infecting such hosts but merely contaminating the host tissue as a result of ingestion (Hill, 1982). Turbot (*Scophthalmus maximus*) and Atlantic halibut (*Hippoglossus hippoglossus*) are both susceptible to IPNV, and in these hosts the virus is pathogenic, causing both pathology and mortality (Mortensen, Evensen, Rodseth, Hjeltnes, 1993, Biering, Nilsen, Rodseth, Glette, 1994).

1.2.8. Post-smolt risk factors

IPN is often considered a disease of fry and fingerling salmonids, but increasingly smolts and post-smolts have been affected (Smail *et al.* 1992, Jarp, Gjerre, Olsen and Bruheim, 1994, Jarp, Taksdal and Torund, 1996). Smail *et al.* (1992) suggested that IPNV might be transmitted to new smolts by a marine vector. However Jarp *et al.* (1994) carried out an epidemiological survey of IPN in post smolts in Norway and noted that if smolts from more than two freshwater hatcheries were mixed at the seawater site the risk of IPN increased by 2.9 compared to farms purchasing from one or two hatcheries. Additionally the risk of IPN was related to the location of the site and the age, newer farm sites were shown to have a higher incidence of IPN than older farm sites. This does not support the theory of a marine vector. Jarp *et al.* (1996) suggested

that it is likely the infection is carried with the smolt to the sea site. Smail *et al.* (1995) examined failed smolt syndrome in relation to IPNV mortalities in the Shetland Isles and fish with high IPNV titres were found to have a poorer condition factor. However the causal mechanisms triggering outbreaks of IPN after transfer to seawater are not known. It is likely that disease is precipitated by a combination of environmental and behavioural factors, especially strong competition for food, acting in competition with viral infection (Smail *et al.*, 1995). Stressors such as handling and transport may lead to an increased susceptibility to disease and increased mortality during the first months of the seawater stage (Jarp *et al.*, 1994). This is in agreement with Roberts and McKnight (1976) who showed that stress could induce a recurrence of IPN in latent carrier rainbow trout.

1.2.9. Control

Control methods for IPN have proven to have only a slight effect on resistance of fish to IPN. Chemotherapy with substances such as polyvinylpyrrolidone-iodine (Economon, 1973) and virazole (Savan and Dobos, 1980) have been tried and found to be costly and relatively ineffective. Avoidance is the most cost effective control measure for IPN, although it is rarely possible.

There have been attempts to produce an IPN vaccine, however this has been problematic and production costs are high. A peptide vaccine has been produced against IPNV (Christie, 1997) but is not commercially available. An inactivated IPNV vaccine is available for use in Norway (Pettersen, 1997) however problems can arise such as inactivated virus leading to a carrier state (Bootland *et al.*, 1991, Christie, 1997). A sub-unit vaccine is also available in Norway using recombinant VP2 added to the

triple vaccine for furunculosis, vibriosis and cold water vibriosis (Frost and Ness, 1997). Although all these vaccines produce a good antibody response in fish experimental challenge to examine their efficacy has not proven possible, since experimental challenges with IPNV do not produce pathology or mortality (Sadasiv, 1995). Coho salmon (*Oncorhynchus kisutch*) are considered to be refractory to IPN (Chevassus and Dorson, 1990) and recently there have been attempts to produce IPN resistant strains of fish.

1.3. Genetic Markers

1.3.1. Importance of genetic markers

To manage a biological resource effectively researchers must identify the level of genetic variation within and between populations (O'Connell and Wright, 1997). Genetic variation is the basic resource of any successful commercial or rehabilitation culture programme (Allendorf and Ryman, 1987). More sophisticated selection approaches require knowledge of the pedigree of the selected population (Herbinger, Doyle, Pitman, Paquet, Mesa, Morris, Wright and Cook, 1995), however individual tagging of fish at the fry stage is not easily achieved in aquaculture (Harris, Bieger, Doyle and Wright, 1991). In breeding programmes families must be reared for long periods in separate cages, tanks or ponds until they can be physically tagged at the family or individual level (Doyle and Herbinger, 1994). For this reason a DNA profiling approach would permit the design of increased selection regimes with a minimal loss of genetic variability on farms without specialised facilities (O'Connell and Wright, 1997).

1.3.2. History of genetic markers

Initially studies on molecular phenotypes used blood group polymorphisms to discriminate between populations of fish (Sick, 1961), however interpretation was difficult and research turned to histochemical staining for specific proteins. In the 1960's studies involved proteins such as haemoglobin and transferrin (Ferguson, 1994). Later histochemical staining was used in conjunction with starch gel electrophoresis and permitted the detection of allozyme variation (Harris and Hopkinson, 1976). This provided the first readily applicable simple genetic markers for large-scale studies on natural populations (Ferguson, Taggart, Prodohl, McMeel, Thompson, Stone, McGinnity and Hynes, 1995). One of the main limitations of protein variants as genetic markers is the low level of polymorphisms in some species and populations (Ferguson *et al.* 1995). The need for a set of unique alleles that have been fixed in a stock specific manner prompted the search for other types of genetic markers (Davidson, Birt and Green, 1989).

Mitochondrial DNA (mtDNA) became popular for genetic studies because of its small size and its rapid accumulation of mutations (Davidson *et al.* 1989). MtDNA has a maternal mode of inheritance and exhibits a lack of recombination, which offered advantages for population studies (Ferguson *et al.* 1995). A significant disadvantage is that mtDNA is usually treated as a single character and does not always permit the examination of independent loci as allozyme studies do (Ward and Grewe, 1995). Work then focused on using restriction fragment length polymorphism (RFLP) analysis (Lansman, Shade, Shapira and Avise, 1981). RFLP analysis assesses variation occurring within a species in the length of DNA fragments generated by a specific restriction enzyme (Park and Moran, 1995). Most RFLP's are not particularly

informative since there are only two alternatives, the individual either has the restriction site or they don't (Wills, 1992). Whilst RFLP's can be very useful for population analysis (Pogson, Mesa and Boutilier, 1995) they have largely been replaced by a new marker system variable number tandem repeats (VNTR).

Research is now focused on VNTR loci examining both mini and microsatellites. In 1985 Jeffreys Wilson and Thein reported the development of multilocus DNA fingerprinting by Southern blot detection of hypervariable minisatellites loci. Initially research concentrated on multilocus DNA fingerprinting, length variation is surveyed at many VNTR simultaneously (O'Reilly and Wright, 1995). Multilocus DNA fingerprinting gave complex results and often both members of allelic pairs at individual loci could not be identified (O'Reilly and Wright, 1995). This prompted the move to single locus VNTR markers where allelic variation is surveyed at individual VNTR loci. Taggart and Ferguson (1990) were the first to use minisatellites in single locus DNA fingerprinting. Minisatellites give a high level of resolution and have been useful in selection programmes where identification of parentage is required (O'Connell and Wright, 1997). However minisatellites do have disadvantages. Mutation mechanisms at minisatellites are very complex, involving intra-allelic duplication or deletion and also inter-allelic recombination and gene conversion (O'Reilly and Wright, 1995). This leads to alleles that may not differ in size by a discrete unit length and this can make accurate scoring of alleles very difficult (O'Connell and Wright, 1997). More recently research has turned to microsatellites where DNA analysis can be performed from tissue such as fins and therefore does not necessitate the sacrifice of organisms as allozyme studies do (Wenburg, Olsen, Bentzen, 1996).

1.3.3. Microsatellites

Dispersed throughout the genome of most eukaryotic organisms are tandemly repeated blocks of DNA of identical or similar sequence (O'Reilly and Wright, 1995). Repetitive DNA is usually classified into two categories: 1) satellite DNA which is composed of large numbers of tandemly repeated sequences and can therefore be isolated from the bulk DNA by centrifugation in a caesium chloride gradient, and 2) interspersed repeated DNA (Estoup, Presa, Vaiman and Guyomard, 1993). Mini and microsatellites belong to the second category. Minisatellites discovered by Bell, Selby and Rutter in 1982 are tandem repeat units of 10-64 bp, with lengths ranging from 0.1-7 kb (Jeffreys *et al.*, 1985). Microsatellite DNA, discovered accidentally, consists of repeating units of 1-5 bp (Jarne and Lagoda, 1996), and are also termed short tandem repeat (STR) DNA (Edwards, Cintello, Hammond and Caskey, 1991). Microsatellites can be tens to hundreds of base pairs of DNA in length (Park and Moran, 1994), and can be composed of different types of repeats, e.g. a GT repeat adjacent to or interposed with GA repeats (Wright and Bentzen, 1994). Microsatellites can be di, tri or tetra nucleotide repeating blocks flanked by regions of non-repetitive DNA (Tautz, 1989). They may be classified into 3 families: pure, compound and interrupted repeats (Jarne and Lagoda, 1996). For example:

Pure	CACACACACACACACACACA
Compound	CACACACACAGAGAGAGAGA
Interrupted	CACATTCACACATTCATTCATT

Microsatellites are highly abundant in eukaryotic genomes; arrays of $(GT)_n$ repeats were estimated to occur on average every 7kb in Atlantic cod (*Gadus morhua*)

(Brooker, Cook, Bentzen, Wright and Doyle, 1994) and 24-35 kb in Atlantic salmon (McConnell, O'Reilly, Hamilton, Wright and Bentzen, 1995b). This figure was re-evaluated with unpublished data and reported in O'Reilly and Wright (1995) as 11-56 kb (Wright pers. comm.).

Tautz (1989) first demonstrated polymorphism at microsatellite loci, since then it has been found that a high proportion of microsatellite loci surveyed in fish are polymorphic (O'Reilly and Wright 1995). For example of 7 loci surveyed in Atlantic cod by Brooker *et al.* (1994), 6 of these were polymorphic. In Atlantic salmon 4 loci were surveyed by McConnell, Hamilton, Morris, Cook, Paquet, Bentzen and Wright (1995a) and all were found to be polymorphic. Expected heterozygosities are well above 50% in general, although compound and interrupted loci tend to be less polymorphic (Jarne and Lagoda, 1996). The informativeness of microsatellite markers increases with increasing numbers of repeats (Weber, 1990). Brooker *et al.* (1994) compared microsatellites in cod to those of mammals, it appears that microsatellites in teleost fishes differ significantly in length and composition from those of mammals. Microsatellite arrays seem to be highly susceptible to length mutation (Wright and Bentzen 1994). The mutational process thought to account for length changes is slipped strand mis-pairing or slippage during DNA replication (Wright 1993). This increases or decreases the current number of repeats by one unit (Jarne and Lagoda, 1996), resulting in high levels of heterozygosity within microsatellites.

1.3.4. Applications of microsatellites

The use of microsatellites can be applied to many molecular genetic studies. The high information content of microsatellite loci relative to more standard genetic markers

suggests great utility to a variety of fisheries and management issues (Scribner, Gust and Fields, 1996). Microsatellites are ideal genetic markers because they are abundant, show varying degrees of allelic variation and can be rapidly assayed by PCR from minute amounts of tissue (McConnell *at al.* 1995b).

The vast majority of fish species and strains reared globally are relatively unimproved for commercially important traits such as growth rates and disease resistance (O'Connell and Wright, 1997). Most traits of commercial importance are polygenic (controlled by more than one locus) and are referred to as quantitative trait loci (QTL) (O'Connell and Wright, 1997), to map QTLs a linkage map is required. The properties of microsatellites make these sequences very useful as markers for genetic mapping and identity control (Slettan, Olsaker and Lie, 1993). The implications for aquaculture are such that microsatellite loci may assist in selective breeding and show proof of breeding levels or strain superiority (Lie, Slettan, Grimholt, Lundin, Syed and Olsaker, 1994).

DNA fingerprinting has proven to be a powerful tool for identifying individuals and the pedigree of communally reared fish (Harris *et al.* 1991). The highly variable nature of microsatellites makes these markers particularly suited to investigation of kinship relationships and paternity analysis (O'Connell and Wright, 1997). Herbinger *et al.* (1995) established pedigrees in mixed aquaculture populations of rainbow trout based on genetic profiling data from microsatellite markers. Parentage was established using four or five microsatellite markers, and 91% of the fish could be traced back to one or two parental couples. Doyle and Herbinger (1994) developed within family selection procedures using microsatellite markers, it was designed to increase selection intensities while minimising levels of inbreeding. McConnell *et al.* (1995b) demonstrated the

utility of microsatellites in population genetics, and were able to discriminate clearly between Canadian and European Atlantic salmon stocks based on unique alleles at two loci. Tessier, Bernatchez, Presa and Angers, (1995) analysed genetic diversity in landlocked Atlantic salmon in Lake Saint-Jean, Quebec, using allozymes, mtDNA and microsatellites. MtDNA proved to be the most discriminating marker between wild populations, but showed little diversity. Microsatellites were most useful in detecting variation between populations and changes in allelic composition between wild and hatchery stocks.

McConnell *et al.* (1995b) demonstrated that the primers developed for three microsatellites in Atlantic salmon also amplified presumably homologous microsatellite loci in a wide range of salmonine species. Estoup *et al.* (1993) noted that primers developed for microsatellite loci in brown trout could also amplify loci in rainbow trout. The reason for this is that particular microsatellite loci and their flanking regions are conserved in related species (Olsen, Wenburg and Bentzen, 1996).

An advantage of the use of microsatellites is the use of semi-automated systems to genotype large numbers of individuals, for example using Perkin-Elmer ABI gene sequencers. This also allows for multiplexing allele fragments from more than one locus from an individual in one lane of an electrophoresis gel (Olsen *et al.* 1996). To increase efficiency further it is possible to co-amplify several microsatellite loci by PCR in a single tube (O'Reilly, Hamilton, McConnell, Wright, 1996). Not only does this mean the use of in lane size standards and therefore possibly increased accurate detection of alleles but it also allows for increased throughput of samples and savings in

consumables (Ziegle, Su, Corcoran, Nie, Mayrand, Hoff, McBride Kronick, Diehl, 1992).

1.4. Genetics of disease resistance

Selection for improved disease resistance in applied breeding programmes may reduce mortality rates in commercial fish farming, the basis for this selection is genetic variation in disease resistance (Lund, Gjedrem, Bentsen, Eide, Larsen, Roed, 1995b). In most studies QTLs with large additive effects have been found (Falconer and Mackay, 1996), additive genetic variance results from the average effect of substituting one allele of a single locus for an alternate form (Parsons, 1998). Additive genetic effects are transmitted directly from parent to offspring. Selection will also affect non-additive gene effects, non-additive gene effects may be caused by interactions between alleles within a locus (dominance) or between alleles in different loci (epistatic interactions) (Bentsen, 1994). Dominance and epistatic effects are not predictably passed from parent to offspring because they are the result of specific allele combinations, these are broken up by recombination and may not be reformed in the progeny (Parsons, 1998). Selection is generally for traits that are affected by additive gene effects, such as disease resistance because these are more accurate predictors.

The relative importance of heredity in determining phenotypic values is called the heritability of a character; the most important function of heritability is its predictive role. It estimates the amount of genetic control of a characteristic, heritability is based on additive effects hence it also indicates the likelihood that traits can be passed onto the next generation and may predict the performance of successive generations

(Parsons, 1998). Heritability (h^2) is defined as the ratio of additive genetic variance (V_A) to phenotypic variance (V_P):

$$h^2 = V_A/V_P$$

(Falconer and Mackay, 1996)

The genetic control of resistance to disease is highly complex and involves the interactions of many systems of the body; one of the most important is the immune system (Roed, Fjalestad and Stromsheim, 1993). Selection for disease resistance may be carried out using direct selection by recording survival or indirectly using correlated traits, such as immune parameters (Fjalestad, Gjedrem and Gjerde, 1993). This requires challenging fish with a disease agent in closed facilities, often these are performed on small fish over a short period of time (Fjalestad *et al.* 1993). To be useful as an indirect selection parameter a marker should show genetic variation and be genetically correlated to disease resistance (Lund *et al.* 1995b). Indirect selection for survival based on a correlated trait with no economic value results in a much lower response in survival compared with direct selection; the genetic correlation between survival and the correlated trait must be large if indirect selection is to compete with direct selection (Fjalestad *et al.* 1993). If genetic correlation between the traits is high the response will increase considerably. Examining underlying resistance markers such as lysozyme and complement allows the identification of factors responsible for differential survival (Marsden, Freeman, Cox and Secombes, 1996). While there have been a few studies on improving disease resistance in Atlantic salmon to furunculosis (Gjedrem, Salte, Gjoen, 1991, Lund, Chiayvareesajja, Larsen, and Roed, 1995a, Lund *et al.* 1995b, Marsden *et al.* 1996) little research has been carried out on improving disease resistance to IPN using immune markers.

1.5. Humoral Immune Mechanisms involved in Disease Resistance

1.5.1. Lysozyme

There are various immune parameters that may possibly be used as markers for disease resistance, one of which is lysozyme. Lysozyme has been detected in the serum, mucus and other tissues of many fish (Fletcher and White, 1973, Murray and Fletcher, 1976, Siwicki and Studnicka, 1987, Mock and Peters, 1990). It occurs mainly in neutrophils and monocytes of fish with small amounts detectable in macrophages (Ingram, 1980). Lysozyme is a mucolytic enzyme attacking structures containing β1-4 linked N-acetylmuramamine and N-acetylglucosamine (Alexander and Ingram, 1992). Lysozyme is thought to have anti-bacterial and anti-viral properties and may act on viruses by promoting phagocytosis as an opsonin or by directly activating leucocytes and macrophages (Jolles and Jolles, 1984).

1.5.2. Interferon

Interferons are cellular proteins that inhibit virus replication (Alexander and Ingram, 1992). Interferon synthesis can be induced by a variety of microorganisms and substances such as nucleic acids, bacterial endotoxins and synthetic polyanions (Ingram, 1980). Interferon, hence its name, interferes with viral replication and growth whilst not directly affecting the particles themselves, its action is directed against the intracellular phases of the virus growth cycle by damaging RNA translation and obstructing protein synthesis, and therefore viral replication (Ingram, 1980). De Kinkelin and Dorson (1973) identified interferon in rainbow trout by its specific protective effect upon cells and its inhibition of virus replication.

Fish have been shown to have two types of interferon, IFN-αβ and possibly IFN-γ (Gravell and Malsberger, 1965, Sigel, 1967, Graham and Secombes, 1990). It is thought that the two interferon's bind to different receptors activating genes and the production of proteins responsible for the activities of interferons (Alexander and Ingram, 1992). Graham and Secombes (1990) demonstrated the production of macrophage activating factor (MAF) by rainbow trout leucocytes after mitogen stimulation. Since IFN-γ is a MAF and the MAF from rainbow trout leucocytes conferred resistance to IPN virus it is probably IFN-γ. IFN-γ activates cells involved in the immune response such as natural killer cells (nonspecific cytotoxic cells in fish) and macrophages (Yano, 1996). Sigel (1967) suggested that interferon compensated for the slow specific immune responses of fish, and played an important role in recovery of fish from viral infections. However, Dorson, De Kinkelin and Torchy (1992) demonstrated interferon synthesis in rainbow trout fry and noted that it coincided with viraemia. In the totally resistant fish no significant interferon activity could be detected.

1.5.3. Complement

Fish have a serum protein system comprising of a large number of complement components (Sakai, 1992). Bony fish possess a complement system similar to that of mammals, it consists of two pathways, the classical pathway and the alternative pathway. The classical pathway is activated by antigen-antibody complexes, the first component is C1 followed by a cascade of interactions of C4, C2, C3, C5, C6, C7, C8, and C9 (Yano, 1996). C5 activates the last components, C8 and C9 producing a large complex causing lysis of target cells (Sakai, 1992). C3 enhances the phagocytic ability of macrophages and neutrophils and acts as an opsonin (Manning, 1994), while C5 is a chemotactic factor for neutrophils and can act as an anaphylatoxin factor for mast cells

(Yano, 1996). The alternative pathway is triggered by a variety of substances such as bacterial endotoxins and polysaccharides, without mediation by antigen-antibody complexes (Sakai, 1992). C3 is directly activated by these substances and leads to cell lysis (Yano, 1996).

The complement pathway may have an anti-viral function. Sakai, Suzuki and Awakura (1994) demonstrated that complement is capable of inactivating pathogenic fish viruses. Rainbow trout and masu salmon (*Oncorhynchus masou*) fry sera showed no haemolytic activity and proved to be much more susceptible to both IPN and IHN (infectious haemopoietic necrosis) viruses than chum salmon (*O. keta*). Virus solutions were incubated with fresh normal serum for 20 hours and showed a reduction in the virus titre. Upon heat inactivation of serum the anti-viral activity was reduced (Sakai, 1992), suggesting complement may have a role in anti-viral defence.

1.5.4. Antibodies

Initial characterisation of fish antibody structure led to the general supposition that fish immunoglobulin (Ig) was comparable to mammalian IgM (Kaattari and Piganelli, 1996). However it is now known that fish have isotypic (structural) differences among Igs (Killie, Espelid and Jorgensen, 1991) which are likely to have different functions in the immune response.

The most direct effect of the interaction of an antibody with its antigen is the act of blocking a critical function of the antigen e.g. a receptor or enzymatic active site (Kaattari and Piganelli, 1996). These antibodies are known as neutralising antibodies. In this way the invaded host can respond to infection with for example IPNV by

production of specific antibodies against the virus surface (Sadasiv, 1995). Havarstein, Endresen, Hjeltnes, Christie and Glette (1990) demonstrated the production of antibody in response to IPNV infection in Atlantic salmon, the antibodies were considered likely to be neutralising antibodies to VP2. Research with monoclonal antibodies suggests that the majority of neutralising antibodies against IPNV are to VP2 (Caswell-Reno, Reno and Nicholson, 1986).

Antibody is also capable of promoting phagocytosis of the antigen by a phagocyte and this is known as opsonisation. Although this is mostly used with bacteria, fungi and parasites, target molecules do not need to be antibody, this may also occur by the activation of complement.

1.6. Stress and Immune Response

Stress may influence the ability of fish to resist pathogenic insults by affecting both the specific and non-specific immune system (Schreck, 1996). Stress caused by transportation and differences between rearing and stocking environment together with elevated water temperatures at the time of release in spring, increases the disease susceptibility of fish (Muona and Soivio, 1992). A substantial body of literature now exists that indicates that corticosteriods secreted in response to stress are partly responsible for the suppression of immunocompetence observed in stressed fish (Barton and Iwama, 1991). One of the main mechanisms involved in stress induced reduction in disease resistance is a suppressive effect on numbers and function of circulating lymphocytes (Ellsaesser and Clem, 1986). Negative effects of stress on disease resistance appear to be due to the depression of antibody synthesising capability via effects on lymphocytes (Schreck, 1996). This has been demonstrated to occur in among

others striped bass stimulated with corticosteroid injection and infected with IPNV (Wechsler, McAllister, Hedrick and Anderson, 1986).

1.7. Genetic Variation in Selection Parameters

1.7.1. Survival/Mortality

Mortality is a very complex trait and commonly has very low heritability (Gjedrem, 1983). Various authors have examined mortality/survival heritabilities. Gjedrem and Aulstad (1974) found significant differences in resistance between river strains of salmon parr to *Vibrio anguillarum* and suggested that the differences were partly heritable. Amend and Nelson (1977) examined the differences in susceptibility of sockeye salmon (*Oncorhynchus nerka*) to IHN virus, they found large differences in mortality between families, ranging from 52% to 98%, suggesting some natural selection for resistance to IHN had occurred. Both studies showed significant differences between full and half-sib families. Gjedrem *et al.* (1991) examined variation in susceptibility of Atlantic salmon to furunculosis and demonstrated that heritability of mortality was high, $h^2 = 0.48 \pm 0.17$ for sires and 0.32 ± 0.10 for dams. Standal and Gjerde (1987) measured survival in Atlantic salmon during the sea rearing period when infected with *Vibrio* spp., heritability was found to be high but there was great variation between year classes and sires.

Silim, Elazary and Lagace (1982) found variations in the susceptibility of IPN between three strains of brook trout where mortalities ranged from 30.9 to 72.3%. Okamoto, Tayama, Kawanobe, Fujiki, Yasuda, Sano (1993) reported a rainbow trout strain (RT201) to be resistant to IPN after years of natural selection. Outbreaks of IPN occurred regularly on the fish farm so survivors were naturally selected IPN resistant

fish. After finding IPNV in the sexual fluids of broodstock the fish were challenged several times a year for 8 years and resistance to IPN was found to be stable with the character persisting even without exposure to natural disease pressure. However the RT201 strain still exhibited some mortalities to IPN indicating that RT201 had not acquired complete resistance to IPN. Ramstad, Taksdal and Korsvoll (1997) noted that mortality in Atlantic salmon fry varied between families (1.3% to 82%) suggesting a possible genetic resistance in certain families of Atlantic salmon.

1.7.2. Immune Parameters

Various associations between immune responses and disease resistance in fish have been reported, however this has mainly been in response to bacterial infections (Refstie, 1982, Chevassus and Dorson, 1990, Roed, Fjalestad, Larsen and Midthjel, 1992). Roed *et al.* (1992) examined genetic variation in haemolytic activity of complement (both spontaneous and antibody dependent) in families of Atlantic salmon. Considerable variation in haemolytic activity was detected, suggesting additive genetic variation of the trait that may be considered as a marker for disease resistance.

Lund *et al.* (1995b) examined genetic variation in haemolytic activity, antibody and lysozyme activity in Atlantic salmon challenged with *Aeromonas salmonicida.* A significant genetic variation in lysozyme activity and an association between low lysozyme activity and high survival rates was demonstrated. Lund *et al.* (1995b) suggested that lysozyme may be a candidate for use in indirect selection to improve resistance against furunculosis, however neither antibody or SH activity were thought to be promising marker traits to improve disease resistance, contradictory to Roed *et al.* (1992) and Roed *et al.* (1993). Lund *et al.* (1995a) examined antibody response as in

indirect marker for improved disease resistance to furunculosis in Atlantic salmon. Antibody alone was not thought to be a very promising marker, however when examined with survival in a regression model the effect of antibodies against the A-layer protein was highly significant.

Balfry, Shariff, Evelyn and Iwama (1994) studied survival and immune response in coho salmon and tilapia (*Oreochromis niloticus*) infected with *Vibrio* spp.. In both species of fish the strains exhibiting the highest survival also possessed a more active respiratory burst, plasma lysozyme activity and increased differential leucocyte counts. Gjedrem and Gjoen (1995) studied differences in susceptibility of Atlantic salmon to *A. salmonicida*, *Renibacterium salmoninarum* and *V. salmonicida*. Heritabilities were found to be low, the highest for BKD (0.23), and the lowest for cold water vibriosis (0.13), selection to develop disease resistant strains of Atlantic salmon was suggested.

Marsden *et al.* (1996) demonstrated differential susceptibilities to furunculosis in Atlantic salmon at farm sites, mortalities ranged from 0.5% to 42.5%, indicating a large phenotypic variation. Non-specific phagocyte functions such as respiratory burst activity and migration were not found to be significantly different between resistant and susceptible families. Both complement and non-α_2 macroglobulin antiprotease activity were found to be higher in families of salmon resistant to *A. salmonicida*.

1.8. Summary

In light of the problems in controlling IPN it is highly desirable to obtain disease resistant strains of fish. This is a long term goal and preliminary evidence suggests that different families of Atlantic salmon do exhibit varying degrees of resistance to viral

diseases such as IPN. The reasons for this varying resistance are unknown but may be genetic and are likely to lead to differing abilities to elicit an immune response to the virus between families.

1.9. Objectives

1. To adapt a previously developed mutliplex PCR to fluorescent genotyping.
2. To genotype Atlantic salmon susceptible to IPNV in the field.
3. To develop a laboratory based challenge on Atlantic salmon post-smolts for IPNV.
4. Using this to challenge different families of Atlantic salmon and examine differing levels of disease resistance and compare the results with those of natural field challenges.
5. To examine certain aspects of the humoral immune response in Atlantic salmon challenged with IPNV.
6. To assess the sensitivity of various diagnostic techniques for IPNV.

2. General Methods

2.1. Cell culture

IPNV was propagated in Chinook salmon embryo cells (CHSE-214). Cells were maintained in Eagles minimum essential medium (EMEM, without L-Glutamine, Gibco) supplemented with 10 % FBS (foetal bovine serum, Gibco), 2 mM L-Glutamine (Gibco) and 1x non-essential amino acids (Sigma). Cells were cultured at 22°C. Where tissue samples were used media was additionally supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin and 100 µg/ml kanamycin (Gibco). CHSE-214 cells were sub-cultured regularly by washing twice with PBS Dulbeccos (without calcium, magnesium and sodium bicarbonate, Gibco) and cells harvested using trypsin-EDTA (1×, in Hanks Balanced Salt Solution (HBSS) without calcium and magnesium, Gibco), and split to a ratio of 1:3.

2.2. Virus culture

IPNV was cultured using either pre-formed or simultaneous inoculation of CHSE-214 cells. For a pre-formed inoculation the media was removed from a fully confluent flask (Iwaki) of CHSE-214 cells and retained. Enough IPNV suspension was inoculated into the flask to cover the monolayer and the virus adsorbed for 30 minutes at 15°C. After this time the conditioned media was replaced and the cells/virus further incubated at 15°C until full CPE was visible. For a simultaneous inoculation a flask of fully confluent CHSE-214 cells was split to a ratio of 1:3 and virus inoculated at $1/10^{th}$ of the volume of the suspended cells. Cells and virus were incubated at 15°C until full CPE was evident. In both cases a mock flask was used where HBSS (Gibco) was inoculated onto the cells. The viral supernatant was harvested and clarified by centrifugation in a

Denley BR401 refridgerated centrifuge at 1000 g for 10 minutes. Virus was stored in aliquots at -20°C.

2.3. Virus isolation

Virus was extracted from head kidney samples kept on ice for no longer than 24 hours or previously frozen. The kidney was placed into a cold sterile mortar with a small quantity of sterile sand and ground together with a pestle. HBSS (Gibco) containing 2 % FBS (Gibco) was then added to the mortar having been previously calculated by weighing the tissue and then diluting it 1:50. The kidney homogenate and HBSS were thoroughly mixed and placed in a centrifuge tube. The solution was then centrifuged at 1000 g for 15 minutes in a Denley BR401 refrigerated centrifuge. The supernatant was removed from the middle of the tube to avoid fatty deposits at the top and passed through a sterile 0.45 µm filter (Sartorius).

One fully confluent 25 cm^2 flask of CHSE-214 cells was then harvested and re-suspended to a volume of 36 ml in EMEM, 1.5 ml of cell suspension was placed into each well of a 24 well plate (Iwaki). Samples were simultaneously inoculated onto CHSE-214 cells at $1/10^{th}$ the volume of cell suspension in each well. Each sample plus HBSS controls were cultured in quadruplicate. The plate was incubated in a 2 % CO_2 incubator at 15°C for 21 days then at day 7 all samples regardless of CPE were passaged and incubated for 14 days. Samples were stored long term at -20°C.

2.4. Virus titration

Where virus titre only was required the method used is as below, section 2.5. except no antisera were used.

2.5. Neutralisation titration

Antisera produced at the SERAD laboratory against reference strains of IPNV A2 (Sp.) and A3 (Ab.) were diluted using HBSS plus 2 % FBS to give final working dilutions of 1:200 and 1:5000 respectively. Each batch of antisera upon arrival at the virology laboratory at Stirling was titrated using an alpha titration to determine the optimal dilution for usage in neutralisations; the above dilutions were optimal for the batch used in this study. Using a sterile flat bottomed 96 well plate (Iwaki); all wells except the first column were filled with 90 µl of HBSS plus 2 % FBS. Column one, rows one and two, were filled with 100 µl of HBSS, the other rows in column one were filled with 100 µl of test virus. The samples were then titrated 1:10 across the plate and 10 µl discarded from the last column, 90 µl of A3 and A2 antisera were added to duplicate rows containing test virus, the other rows all contained a further 90 µl of HBSS plus 2 % FBS. Virus was adsorbed for a minimum of 30 minutes at 15°C. During this time a 25 cm^2 tissue culture flask (Iwaki) of confluent CHSE-214 cells were harvested. Cells were made up to 12 ml with EMEM, 100µl of cells was added to every well and the plate incubated in a 2 % CO_2 incubator at 15°C for 7 days. Estimation of virus titre was calculated using the Spearman-Karber method for estimation of 50% end points (Karber, 1931).

$$\text{Mean log } TCID_{50} \text{ (m)} = x + \tfrac{1}{2} d - d \sum (r/n)$$

Where x = log of the highest reciprocal dilution

d = log of the dilution interval

r = number of test subjects not infected at any dilution

n = number of test subjects inoculated at any dilution

2.5. Virus purification

Virus was propagated and clarified by centrifugation at 1000 g for 10 minutes. The resulting supernatant was removed and placed into ultra-centrifuge tubes (Beckman) previously disinfected with 70 % ethanol (Fisher), weighed to ensure the tubes were accurately balanced. Tubes were filled to within a couple of millimetres from the top. Viral supernatant was ultra-centrifuged in a Beckman L80 ultra centrifuge at 12,000 g for 35 minutes as an ultra clarification step. The supernatant was removed and placed into clean newly disinfected tubes, weighed and centrifuged at 100,000 g for 1 hour 35 minutes to pellet the virus. The supernatant was then removed and discarded, the viral pellet was re-suspended in 100 µl TNE buffer (Appendix 1) at room temperature for 15 minutes, the tubes sealed with parafilm and left at 4°C overnight. Purified virus was then pooled and stored at -20°C.

2.6. Histology

2.6.1. Tissue sampling, preservation and processing

Atlantic salmon organ samples (liver, kidney, gut and pancreas, heart, gill and spleen) were placed in 10 % neutral buffered formalin (Appendix 1). The tissues were cut to the correct size and placed into coded cassettes, the cassettes were immediately placed into water until processing. Processing was carried out in an automatic tissue processor (Shandon Citadel). The tissues were passed through increasing percentages of alcohol until at absolute alcohol, then through chloroform and molten wax (Appendix 2).

2.6.2. Tissue embedding

After overnight processing the cassettes were placed into the molten wax bath of the histoembedder (Leica). The organs were then embedded in paraffin wax in suitable

moulds and immediately placed onto a cool plate. Once solidified the blocks were removed from the mould and stored at room temperature until sectioning.

2.6.3. Tissue sectioning

Initially the blocks were trimmed to access the tissues, all sectioning was carried out on a Leica 2035 BIOCUT microtome. Soft tissues such as kidney, liver, heart, spleen and pyloric ceaca were placed into distilled water after trimming, hard tissues such as gills were placed in decalcifier for an hour unless immunohistochemistry was planned and then the tissue was also placed into distilled water. Blocks were sectioned at 5 μm, ribbons of sections were floated on distilled water in a water bath at 40°C and the best section slipped onto a glass slide, the slide labelled and left to dry on a drying rack at 40°C. When all the sections had been taken the slides were dried in a drying oven at 60°C overnight.

Slides were stained with haematoxylin and eosin (H&E) and mounted with pertex. See Appendix 2 for exact staining procedure.

2.7. Bacteriology

2.7.1. Isolation

Bacteria were isolated by taking a swab of the kidney, which was then streaked onto tryptic-soya agar (TSA, Oxoid), plus 1.5 % sodium chloride (BDH) plates (Appendix 1). Plates were incubated at 20°C until bacterial growth or for 7 days.

2.7.2. Identification

Cultures were identified using standard bacteriological techniques.

2.7.2.1. Gram's stain

Using aseptic techniques a loopful of sterile saline was placed onto a clean glass slide. A minute quantity of culture was placed on the slide, the culture and the saline were emulsified and spread evenly over the slide. Once the slide was dry the slide was passed through a bunsen flame three times to fix the culture and left to cool. The slide was then covered in 1 % crystal violet (BDH, Appendix 1) solution and left for one minute, washed with distilled water and then covered with iodine (BDH) and left for one minute. The slide was then covered with acetone (Fisher) for 10 seconds and thoroughly washed with distilled water and air dried. The slide was then covered with safranine solution (Sigma, Appendix 1) for two minutes, washed in distilled water and air dried. The slide was then examined microscopically under oil, the size and shape of the culture were noted along with its Gram status.

2.7.2.2. Motility test

Vaseline was placed on all four corners of a coverslip, a loopful of saline was placed into the middle of the coverslip, a minute amount of culture was then emulsified with the saline. A microscope slide was gently lowered onto the coverslip and the slide turned over and examined microscopically using x40 objective.

2.7.2.3. Oxidase test

Oxidase sticks (Oxoid) were touched onto the culture, a colour change from red to purple within thirty seconds indicated the test was positive and the reagent oxidised.

2.7.2.4. 0/129 sensitivity

This test is specifically used to identify *Vibrio* species of bacteria as 0/129 (2, 4-diamino-6, 7-di-iso-propyl pteridine phosphate) is a bacteriostatic agent effective against *Vibrio* sp.. Several colonies of culture were inoculated into 1 ml of sterile saline and the culture suspended by gently shaking. Using a sterile pipette 5 drops of the suspension were dropped onto a TSA plus 1.5% sodium chloride agar plate and spread evenly around the plate using a sterile glass spreader. The plate was left to dry for 1 minute and then one 10 μg disc impregnated with 0/129 (Oxoid) placed one side of the plate and one 150 μg disc (Oxoid) placed on the other side. The plate was inverted and incubated at 20°C.

2.7.2.5. API 20E microbial identification

The API 20E (BioMerieux) test is a microtube system that enables 23 standard biochemical tests to be carried out on a gram-negative bacterial culture. Several colonies of pure culture were placed into 5 ml of sterile saline and the mixture gently shaken to suspend the bacteria. The API 20E test strips were placed inside disposable plastic trays supplied by the manufacturer, which were then filled with distilled water to form a humid chamber. The trays were titled and using a sterile pipette the microtubes were filled with suspension, with three enzymes CIT, VP and GEL the wells along with the microtubes were filled with bacterial suspension. After this inoculation the well section of ADH, LDC, ODC, H_2S and URE tubes were filled with liquid paraffin. The strip was incubated at 20°C for 3 days. After this time I drop of TDA reagent was added to the TDA tube, 1 drop of VP1, then 1 drop of VP2 reagent was added to VP tube and 1 drop of IND reagent was added to the IND tube. The subsequent colour change was scored and compared with the results of known fish pathogens.

2.8. Anaesthesia of fish

Atlantic salmon were anaesthetised using ethyl p-aminobenzoate (Sigma) at a stock concentration of 40 g/l in methanol (Fisher), the stock was diluted 1:1000, 1 ml of ethyl p-aminobenzoate was mixed with 1 litre of seawater. Where fish were to be terminally anaesthetised the spinal cord was also severed.

3. The use of microsatellites to assign Atlantic salmon susceptible to IPNV in the field to a family

3.1. Introduction

IPNV has been recognised as a problem for post-smolt Atlantic salmon since the 1980's. Outbreaks usually occur between 6-8 weeks post transfer to seawater in Scotland, the Shetland Isles and Norway (Krosgrud, Hastein and Ronnigen, 1986, Smail *et al.*, 1992, 1995). Smail *et al.* (1992) reported clinical outbreaks of IPN on two Scottish Atlantic salmon farms in 1987 with mortality higher than 10% per week in certain cages. Smail *et al.* (1995) demonstrated that in 1990 Shetland suffered major outbreaks of IPN and noted that fish exhibited particularly marked IPN pathology unlike anything that had been seen before with very high titres of IPNV. The pattern has changed very little since this time, for this reason Shetland was the ideal location for placing a stock of Atlantic salmon to undergo a natural challenge with IPN. An indication of the variation in disease resistance between families of Atlantic salmon to IPN could be determined by mixing offspring from known parents and placing them at a location where they were likely to be challenged with the virus and monitor mortalities.

Microsatellite markers have been extensively used in the reconstruction of proximal relationships between individuals for many and varied applications (O'Reilly, Herbinger and Wright, 1998). Atlantic salmon microsatellite markers developed by O'Reilly *et al.* (1996) for use in parentage determination were used in this study to assign fish susceptible to IPN to their parents. O'Reilly *et al.* (1996) developed a system using four microsatellites; one di-nucleotide locus, Ssa85 and three tetra-nucleotide loci, Ssa197, Ssa171, Ssa202, all four loci could be co-amplified

(multiplexed) in a single reaction. This system was developed using radioactively labelled primers and PCR products visualised using standard sequencing gels.

The utility of this system was evaluated using different wild populations of Atlantic salmon in three different rivers in Nova Scotia, Canada O'Reilly *et al.* (1996) examined allele frequency, heterozygosity and conformance with Hardy-Weinberg (H-W) equilibrium. Heterozygosity values at the four loci ranged from 80-91%, it was also found using the three populations that the number of alleles at each locus was high and varied from 12-25. To test for H-W equilibrium and Mendelian inheritance three sets of parents with 20 offspring from each were typed and were found to exhibit classic Mendelian inheritance with no significant departures from H-W equilibrium. O'Reilly *et al.* (1998) assigned 800 Atlantic salmon offspring to a set of 12 parents using the same microsatellites and found that 99.5% of the offspring analysed could be assigned unambiguously to one set of parents. For the above reasons this particular set of microsatellites were considered to be very suitable for use in parentage assignment in this study.

3.2. Aims

1. Using a pedigreed stock of Atlantic salmon from Landcatch Ltd. to establish the occurrence of an outbreak of IPN using virus culture and histopathology.
2. To genotype the susceptible fish using microsatellites and assign these fish to pairs of previously genotyped parents.
3. To examine the resulting data for any correlation between family and susceptibility.

3.3. Methods

3.3.1. Fish rearing and natural challenge

Atlantic salmon from single pair matings of genetically known parental stock were maintained in 160 separate family groups' at the hatchery until smoltification. A suitable fish farm was chosen in Out Skerries, Shetland to transfer 39,000 of these Atlantic salmon smolts, known as the sentinel fish, (equal numbers of fish from each family) to seawater in April 1998. The chosen farm had a history of repeated outbreaks of IPNV causing mortality annually 8-12 weeks post transfer to seawater. Fish were transferred by well boat and upon arrival at the farm were distributed into 4 cages, at transfer the fish were approximately 100g in weight. Mortalities were removed from the cages by divers every week until a large increase in deaths was noted (approximately 10 weeks post transfer), mortality collection was then increased to twice a week. Data was collected from 5 dives during a period of 3 weeks in July 1998. Dead salmon were sorted according to state of decomposition, whole fish were weighed, their length measured and a fin clip taken. Fin clips were either placed straight into extraction buffer for immediate DNA extraction at the farm or into absolute ethanol for extraction at a later date.

3.3.2. Tissue sampling

Organ samples were taken from six fish at the fish farm and immediately placed into 10% neutral buffered formalin for histopathology. A swab of the kidney was taken from each fish and streaked onto TSA plus sodium chloride agar. Samples of head kidney were also taken from the same fish for virus culture and kept at 4°C for 24 hours until processing. A fresh sterile scalpel blade was used for each fish. For tissue processing, histopathology, bacteriology, virus culture and identification see Chapter 2.

3.3.3. DNA extraction

DNA was extracted from fin tissue using a modified method of van de Ven, Lanham, Brennan (1996). Fin tissue was placed in 400µl of extraction buffer (Appendix 1) in sterile 1.5ml microcentrifuge tubes (Alpha) and left at room temperature overnight. The tissue was then homogenised using a sterile disposable pestle (Sigma) and vortexed for 5 seconds. The tube was centrifuged in an MSE Micro Centaur at 14000g for 1 minute, and 300µl of the supernatant was removed and placed into a fresh sterile tube. An equal volume of cold (4°C) isopropanol (BDH) was added to each tube, incubated at room temperature for 2 minutes and centrifuged for 10 minutes at 14000g. The supernatant was then discarded, the pellet washed once with 300µl of 70% ethanol (BDH) and centrifuged for 5 minutes at top speed. The supernatant was discarded and the pellet air-dried, DNA was re-suspended in 100µl of TE buffer (Appendix 1) and stored at 4°C until use and at -20°C for long term storage.

3.3.4. Multiplex PCR

The multiplex PCR consisted of four salmon microsatellites, Ssa202, Ssa171, Ssa197 and Ssa85, these microsatellite loci were isolated and specific primers designed by O'Reilly *et al.* (1996). Primers were synthesised at Perkin-Elmer Applied Biosystems and one member of each primer pair was fluorescently labelled. The ABI 377 was able to recognise 4 colours using filter set C, TET (green), HEX (yellow), 6-FAM (blue) and TAMRA (red). The size of alleles in the multiplex indicated the use of GS350 TAMRA size standard (Perkin-Elmer).

The primers were labelled with:

Ssa202- green (TET)

Ssa171- blue (6-FAM)

Ssa197- yellow (HEX)

Ssa85 - green (TET)

Table 3.1: Nucleotide sequences of primers and microsatellites (taken from O'Reilly *et al.* 1996).

Locus	Repeat Motif	Sequence (5'-3')	Allele size range
Ssa85	$(GT)_{14}$	a) GG TGG GTC CTC CAA GCT AC	123-160
		b) ACC CGC TCC TCA CTT AAT C*	
Ssa197	$(GT)_5C(TG)_4TC(TG)_3$ $A(GTGA)_{15}$	a) GGG TTG AGT AGG GAG GCT TG	164-264
		b)TGG CAG GGA TTT GAC ATA AC*	
Ssa171	$(TGTA)_{14}(TG)_7$	a) TTA TTA TCC AAA GGG GTC AAA A	200-250
		b) GAG GTC GCT GGG GTT TAC TAT*	
Ssa202	$(CA)_3(CTCA)_{17}$	a) CTT GGA ATA TCT AGA ATA TGG C	240-276
		b) TTC ATG TGT TAA TGT TGC GTG*	

*fluorescently labelled primer

Due to the marked difference in sizes of alleles at Ssa202 and Ssa85 it was possible to label both sets of primers with TET. Despite the use of a previously optimised PCR the different primer labelling methods, different equipment and DNA quality required optimisation of the PCR. Initially annealing temperature was varied from 50-58°C until an optimum was found. Magnesium chloride ($MgCl_2$) was titrated from 1.5 mM to 5 mM in 0.5 mM increments using primer concentrations of 0.5μM for each locus. Once an annealing temperature was chosen and $MgCl_2$ concentration decided primer

concentrations were gradually lowered until optimum amplification at all loci was obtained. Amplification reactions were carried out in a volume of 10µl containing 1µl PCR buffer, 0.5 units of *Taq* (Promega), 1.5 mM $MgCl_2$ and 200 µM each dNTP (Promega). Primer concentrations were 0.1 µM for 85 a and b, 0.2 µM for 202 a and b, 0.2 µM for 197 a and b and 0.25 µM for 171 a and b.

Thermal cycling conditions were:

10 cycles of: 20 s at 94°C (denaturing)

20 s at 53°C (primer annealing)

20 s at 72°C (extension)

35 cycles of: 20 s at 90°C

20 s at 53°C

20 s at 72°C.

7 minutes at 72°C (final extension)

30 minutes at 60°C (ensures A addition to every copy)

NO 4°C hold

Amplification was performed on a PE Applied Biosystems GeneAmp® PCR System 9700 thermal cycler, in 96 well plates (Elkay).

3.3.5. Electrophoresis

PCR products were separated using a Perkin-Elmer ABI 377 Automated Sequencer. The denaturing acrylamide gels were made by mixing 5.2ml acrylamide:bis 19:1 solution (Biorad) with 27.5ml nanopure water, 0.5g mixed bed resin (Sigma) and 18g urea (Biorad), once at room temperature the mix was then 0.2µm filtered (Whatman) and 5ml 10x TBE (Appendix 1) added through the filter. The solution was then

degassed for 4 minutes; 250µl of freshly prepared APS (ammonium persulfate, Amresco) and 35µl TEMED (Biorad) were added and the mix gently swirled. The gel mix was taken up in a 50ml syringe and injected between 36cm gel plates using the injection device supplied with the sequencer and allowed to polymerise for 2.5 hours prior to use.

The PCR products were diluted 1:10 with nanopure water, 1.5µl of diluted PCR product was mixed with 0.6µl GS350 TAMRA size standard, 0.5µl loading dye (Appendix 1) and 2.4µl formamide (Sigma). Samples were denatured at 95°C for 5 minutes on the PE GeneAmp® PCR System 9700 thermal cycler and held on ice until use. The gel was pre-run for 10 minutes at 1200 volts. After this each lane of the gel was loaded with 1.0µl of the PCR product mix, alternate lanes were loaded to avoid contamination between lanes, the gel was then pre-run for 3 minutes to run the first samples into the gel and then the remaining lanes loaded. Each gel was run for 2 hours at 1200 volts.

3.3.6. Genotyping

The lanes on the gel were tracked manually and the data from each lane extracted and sent to GeneScan™ version 2.1 to be analysed. GeneScan™ then created a project containing all the data from the gel. Analysis was carried out by firstly applying a previously created matrix to the samples to allow the machine to distinguish between the colours. Previously programmed analysis parameters were applied to the data, GeneScan™ performed size calling by using the local southern method, no peak smoothing and called peaks above a height of 50 units. Results were then imported into Genotyper™ which then used two macros written specifically for this multiplex system to further analyse the data. The first macro removed stutter peaks from Ssa85 alleles

and indicated the presence of more than two peaks at any one locus that could then be manually adjusted. The second macro organised data into tabular form ready to be imported into Excel as a spreadsheet.

3.3.7. Parental genotyping

The post-smolts used in this study were hatched in December 1996 from 328 broodstock Atlantic salmon, the parental fish were fin clipped and specific crosses carried out and recorded. Prior to this study the parental fin clips were sent to the Marine Gene Probe Laboratory, Dalhousie, Canada to assess that the parental stock was indeed variable and to such a degree that offspring could be assigned. These samples were genotyped using radioactive labelling techniques, to ensure compatibility of the fluorescent genotyping with radioactive genotyping 100 parents already genotyped in Canada were also run on the PE ABI 377 sequencer.

3.3.8. Parentage assignment

Once in Excel allele sizes for parents were altered to letters and genotypes analysed using Pedigree Analysis - Family Genotype Comparisons, Version 2.03 a program written and kindly provided by Dr. John Taggart (University of Stirling). The resulting output file gives a predictive figure on the percentage of all offspring that could be assigned to a family and within each family the percentage of offspring that could be assigned unambiguously to that family. Offspring genotypes were also altered to letters and parentage assigned using the same programme. Both sets of data, the parents and potential offspring were also analysed using Genepop Version 3.1a to establish allele frequencies and if the data were Hardy-Weinberg equilibrium. Estimation of exact P-

values were calculated using Markov chain parameters, with dememorization set at 2000, testing was carried out in batches of 200 with 2000 iterations per batch.

3.3.9. Parental genotyping of the 1997 parents

In preparation for sampling IPN susceptible fish the next summer the parents of those fish were also genotyped and resulting genotypes analysed using the Pedigree Analysis programme.

3.3.10. Sibling genotyping and parentage assignment

After genotyping the Skerries group of 1996 offspring 64 of their siblings maintained at the hatchery to later become broodstock were also genotyped. The individuals were fin clipped, the samples genotyped and parentage assigned as above.

3.3.11. Reliability testing

To ensure the DNA extraction, PCR, genotyping and parental assignment were all accurate and reliable 4 sets of parents and 6 offspring from each set of parents from the 1998 broodstock were fin clipped. The samples were genotyped and parentage assigned as above.

3.4. Results

3.4.1. Disease signs

3.4.1.1. Mortality

Mortalities were collected at weeks 13, 14 and 15 post transfer to seawater, only 401 salmon were fin clipped during this time because the majority of the fish were too decomposed to sample. The mean condition factor of the fish sampled was 0.8 (standard deviation, 0.21).

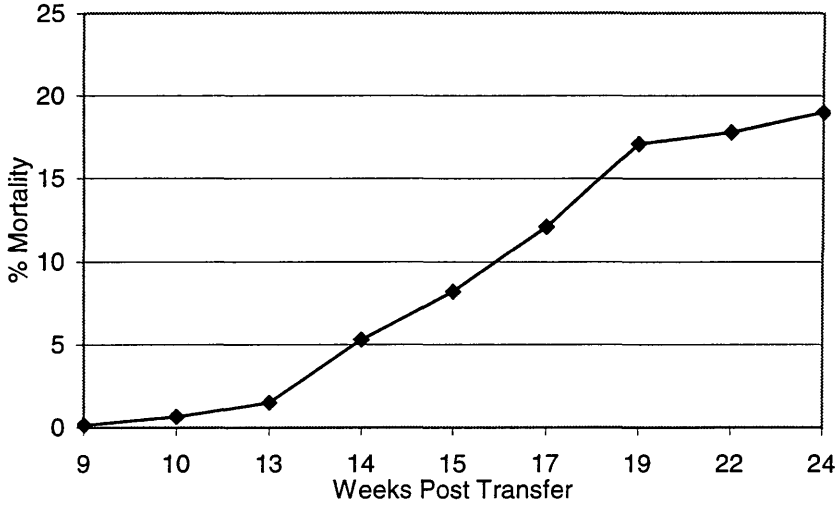


Figure 3.1: Cumulative percentage mortalities in the stock at Out Skerries during an outbreak of IPN.

3.4.1.2. Bacteriology

All fish sampled at the fish farm were found to be infected with *Vibrio* spp., the cultures were very mixed, with no one species appearing to be the most prominent.

Table 3.2: Characteristics of bacterial cultures isolated at the fish farm.

Test	Result
Gram	negative
Shape	curved rods
Size	medium
Motility	none
Oxidase	positive
0/129-10μg	positive
0/129-100μg	positive

The API 20E test was found to be inconclusive and the reactions were not indicative of any one *Vibrio* species.

3.4.1.3. Virology

All the fish sampled were found to be infected with a virus, after a neutralisation titration was carried out the virus was confirmed to be IPNV A2 (Sp.) strain.

3.4.1.4. Histopathology

The gut and pancreas in all fish exhibited varying degrees of IPN pathology (Figure 3.2). Pancreatic pathology included necrosis of fatty tissue surrounding the pancreas, a mononuclear infiltrate into the fatty tissue, release of zymogen granules and pancreatic acinar cell necrosis. The gut contained a catarrhal exudate indicating gut enteritis typical of IPN.

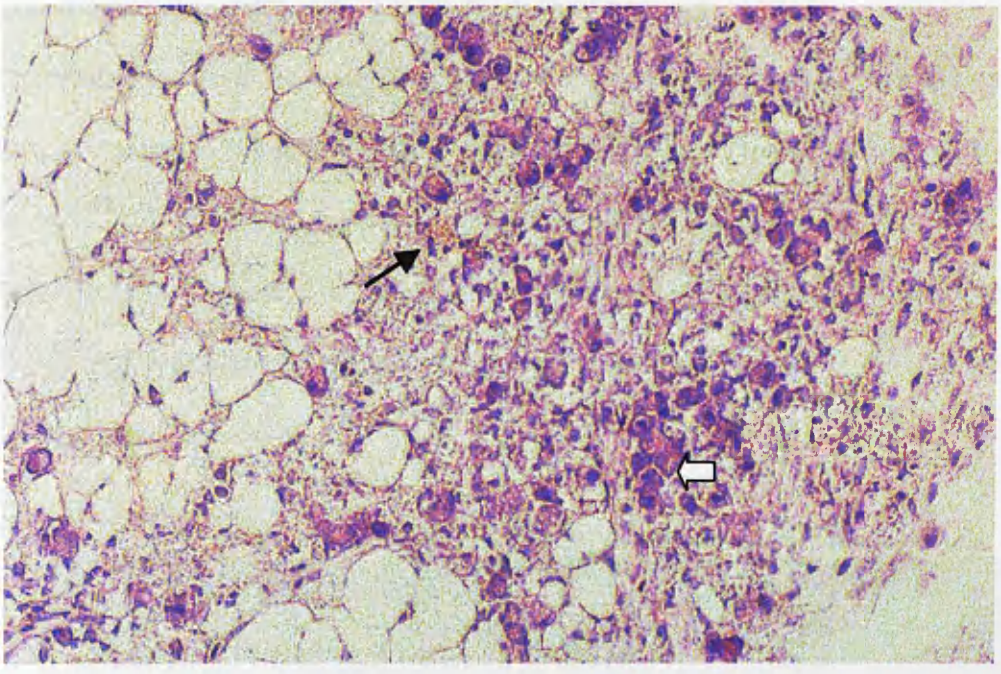


Figure 3.2: IPN pathology in the pancreas of Atlantic salmon stained with H & E. Magnification: x20. Nuclear debris from exocrine cells (solid arrow). Degenerate exocrine cells (open arrow).

3.4.2. Genotyping

3.4.2.1. Gel interpretation

Initial results from the PCR are shown in the gel image below. From this the lanes were tracked using the tracker lane shown. As already mentioned the red bands are the size standard GS350, sizes from bottom to top are 139, 150, 160, 200, 250, 300, 340, 350 bases. Green bands around 100-160 bases are alleles of Ssa85, yellow bands alleles of Ssa197, blue bands alleles of Ssa171 and green bands around 230-300 bases are alleles of Ssa202.

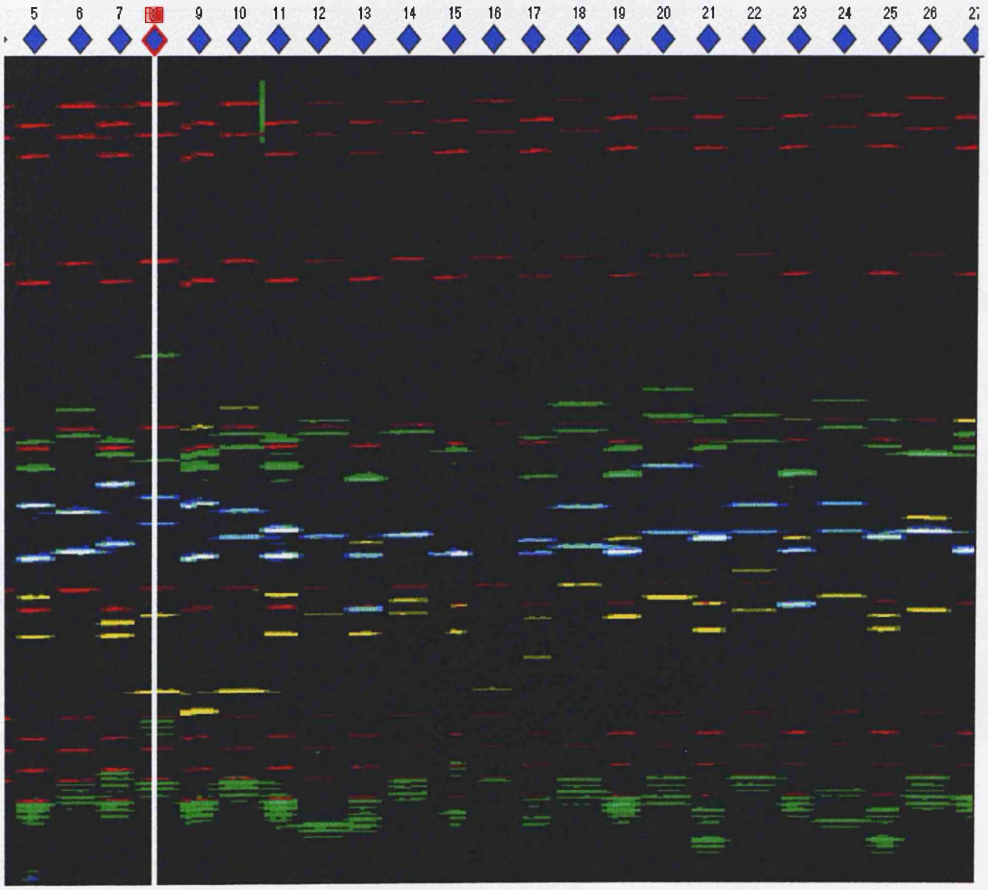
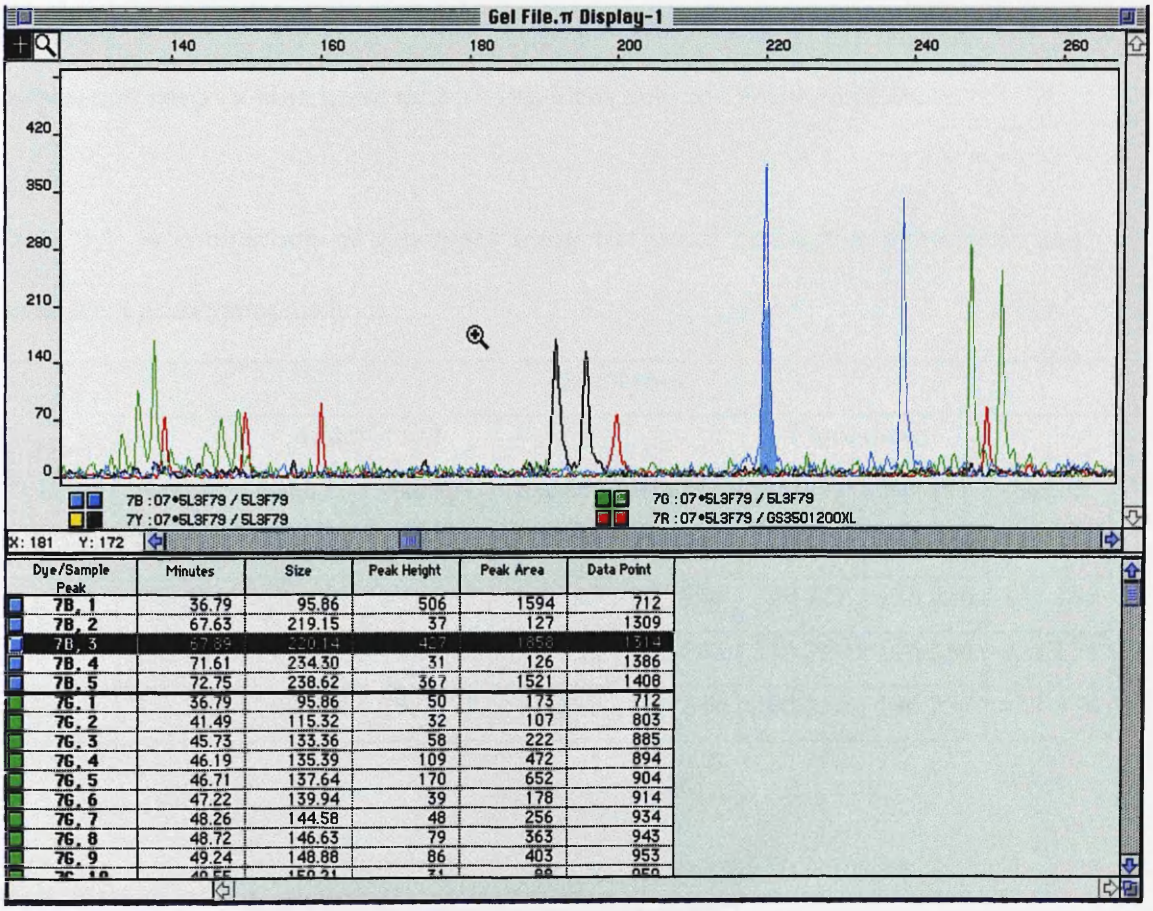


Figure 3.3: Example of gel image to show microsatellites and size standard (red) in each lane and tracking method.

3.4.2.2. GeneScan™ analysis

Once the data from the gel had been extracted and imported into GeneScan™ 2.1 the data was analysed using the analysis parameters mentioned in section 3.3.6. Each lane/sample is represented as an electropherogram as seen below, individual peaks could then be highlighted and sized (yellow is seen as black).



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
7B, 1	36.79	95.86	506	1594	712
7B, 2	67.63	219.15	37	127	1309
7B, 3	67.89	220.14	427	1858	1314
7B, 4	71.61	234.30	31	126	1386
7B, 5	72.75	238.62	367	1521	1408
7G, 1	36.79	95.86	50	173	712
7G, 2	41.49	115.32	32	107	803
7G, 3	45.73	133.36	58	222	885
7G, 4	46.19	135.39	109	472	894
7G, 5	46.71	137.64	170	652	904
7G, 6	47.22	139.94	39	178	914
7G, 7	48.26	144.58	48	256	934
7G, 8	48.72	146.63	79	363	943
7G, 9	49.24	148.88	86	403	953

Figure 3.4: Electropherogram to show the microsatellite profile of one individual and size marker in GeneScan™.

3.4.3. 1996 parental genotyping

Comparison of radioactively labelled genotyping with fluorescent genotyping indicated that although the alleles at two loci were seen as a slightly different size (Ssa202 was two bases larger and Ssa197 was one base larger) this was consistent throughout all the individuals. Alleles at Ssa171 were the same size using both methods. However locus Ssa85, as a di-nucleotide is subject to stutter, this can cause problems with typing the correct allele and for this reason alleles were not consistently different. Therefore all the parental samples were genotyped for this locus only and analysed as above.

Table 3.3: A comparison of genotypes using traditional radioactive genotyping and fluorescent genotyping methods.

	Genotypes							
	Traditional				**Fluorescent**			
Individual	**Ssa202**	**Ssa171**	**Ssa197**	**Ssa85**	**Ssa202**	**Ssa171**	**Ssa197**	**Ssa85**
1	246 246	220 228	195 255	133 139	248 248	220 228	196 256	131 138
2	238 246	220 228	255 255	133 139	240 248	220 228	256 256	131 138
3	238 246	216 220	195 255	125 133	240 248	216 220	196 256	123 131
4	238 246	200 216	195 255	135 139	240 248	200 216	196 256	133 138

3.4.4. Parentage assignment

3.4.4.1. Assignment of the Skerries stock

The Pedigree Analysis programme predicted that 83% of offspring from the 1996 parents could be assigned unambiguously to a family assuming equal numbers of offspring from each family were present. This result indicated that the majority of individuals sampled at Skerries would be able to be correctly assigned to their parents.

In total in Shetland 401 dead or dying fish were fin clipped, from these fish 27 could not be fully genotyped probably due to degraded DNA, a full genotype was required for the assignment programme and consequently these fish were excluded from the analysis. Analysis was carried out on the remaining 374 fully genotyped individuals, however, only 119 offspring out of the 374 could be typed to a family. The programme could find no parental matches for the remaining individuals using the given parental crosses. Analysis was carried out in total on three different occasions, with the data being re-entered into the computer each time to discount data entry errors. Prior to this samples had been run more than once on the ABI sequencer to ensure accuracy, with the same results obtained. Therefore 32% of the individuals sampled were assigned to one or more families, with 25% of the total number of fish genotyped assigned to one family only. A breakdown of family assignment and allele coding can be seen in Appendix 3.

Table 3.4: An example of genotype and family assignment results from the first 10 Skerries offspring mortalities.

Offspring ID	**Genotype Ssa202, Ssa171, Ssa197, Ssa85**	**Families Sharing Genotype**
1	CF, BF, JQ, GI	No match
2	AC, AG, BE, AA	No match
3	AD, CD, EH, CD	No match
4	AC, CG, QQ, CC	91, 100
5	AF, DE, GM, AJ	No match
6	AD, CD, EH, GI	153
8	AD, CE, EH, AA	No match
9	AA, CC, II, KK	No match
10	CI, GI, IQ, GG	93
11	DD, GG, BH, AA	No match

3.4.4.2. Assignment of siblings

After the lack of offspring matches from the Skerries fish DNA was extracted from 100 siblings still at the hatchery, 64 siblings were genotyped (a full gel run), full genotypes were obtained from 44 fish out of the total 64. Although there was an increase in the matches to 64% there were still 36% whose parentage was unaccounted for.

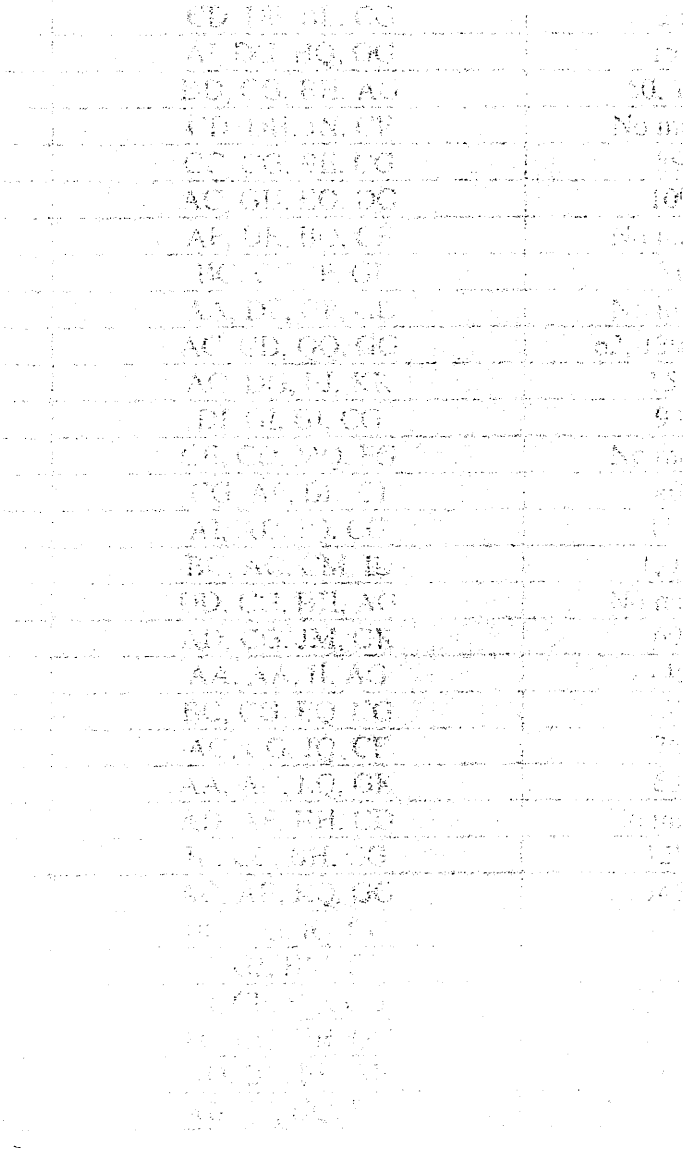


Table 3.5: Results of the family assignment from the Skerries offspring siblings chosen at random for genotyping.

Fish ID	Genotype Ssa202, Ssa171, Ssa197, Ssa85	Families sharing genotype
1	AC, AF, CH, DG	No match
2	AA, DF, HP, GL	No match
3	GG, DF, JJ, CF	No match
4	AI, AD, IJ, GG	145
5	BC, CC, BI, CG	41, 128
6	CC, CG, JJ, CG	No match
8	AF, DD, HL, CK	107
12	CD, AG, FI, CC	No match
14	AC, AF, BQ, AA	No match
16	CD, DF, BL, CC	26
18	AI, DG, HQ, GG	154
19	DD, CG, BH, AG	50, 104
20	CD, DH, JN, CF	No match
23	CC, CG, BE, CG	89
24	AC, GH, EO, DG	109
25	AF, DE, BO, CF	No match
26	BC, CF, JP, GI	31
27	AA, DF, GP, GL	No match
28	AC, CD, QQ, GG	62, 139, 143
29	AC, DG, EJ, KK	151
30	DI, GI, GI, CG	93
31	CE, CD, MQ, FG	No match
33	CG, AF, BL, CI	40
35	AI, AD, JQ, CG	111
37	BC, AC, CM, IL	1, 15
38	DD, CG, HH, AG	No match
40	AD, CG, JM, GK	69
44	AA, AA, IJ, AG	145
47	BC, CG, EQ, CG	157
49	AC, CG, JQ, CF	76
50	AA, AC, LQ, GK	63
51	AD, AF, HH, CD	No match
61	BI, CC, BH, CG	128
62	AC, AE, KQ, GG	143
76	BC, DG, IQ, CC	100
83	EI, GJ, HM, FG	No match
88	CI, CF, HQ, CG	128, 146
95	AC, CC, HH, GG	56, 144
99	AD, BE, BF, AF	No match
100	AB, CJ, GQ, FN	No match

3.4.4.3. Reliability Testing

Table 3.6 (overleaf) shows the offspring of the first three families genotyped from the 1998 broodstock and offspring were assigned to the correct parents. The offspring reported to belong to the fourth set of parents did not match, although the offspring did all appear to be from the same family.

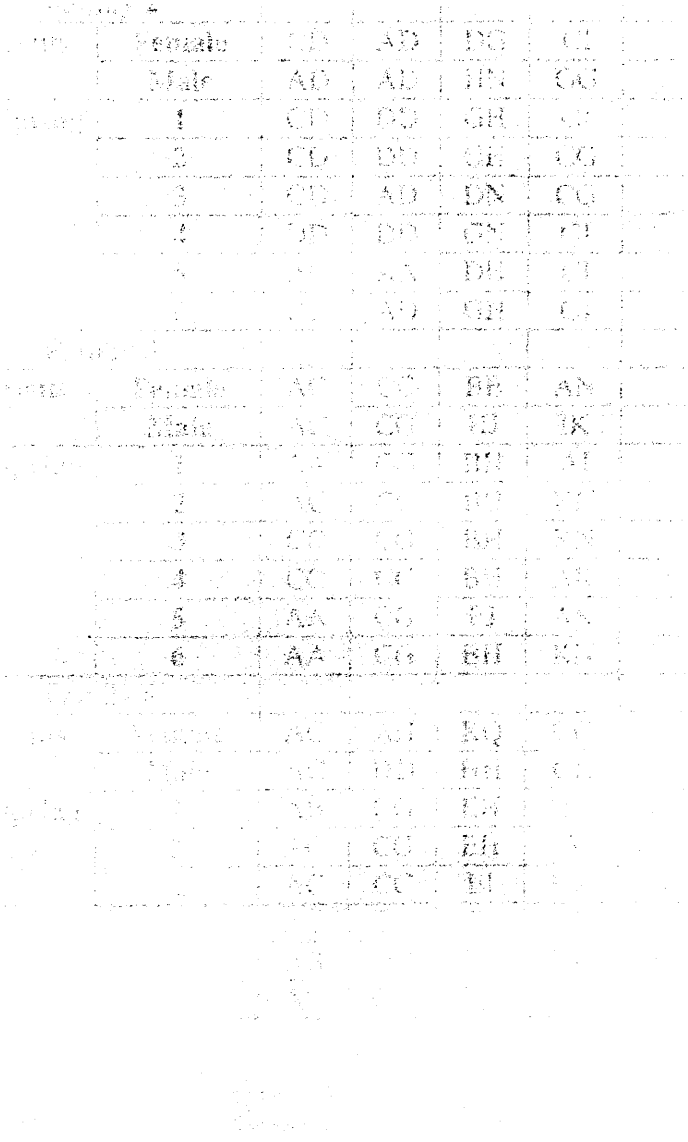


Table 3.6: Results of parentage assignment in the 6 families of fish used to test the reliability of the genotyping system.

Family		Genotype				Family Assignment
		202	171	197	85	
Family 1						
Parents	**Female**	CD	CD	HJ	CG	
	Male	AD	CF	BQ	GN	
Offspring	**1**	DD	CC	HQ	GN	1
	2	AD	CF	BJ	CN	1
	3	CD	CD	BH	CN	1
	4	AC	CD	HQ	CN	1
	5	CD	CD	JQ	CG	1
	6	Individual not fully genotyped				
Family 2						
Parents	**Female**	CD	AD	DG	CI	
	Male	AD	AD	HN	GG	
Offspring	**1**	CD	DD	GH	GI	2
	2	CD	DD	GH	CG	2
	3	CD	AD	DN	CG	2
	4	DD	DD	GN	GI	2
	5	AC	AA	DH	CI	2
	6	DD	AD	GH	GI	2
Family 3						
Parents	**Female**	AC	CC	BE	AN	
	Male	AC	CG	HJ	IK	
Offspring	**1**	AA	CG	BH	AI	3
	2	AC	CC	BH	KN	3
	3	CC	CG	BH	KN	3
	4	CC	CC	BH	AK	3
	5	AA	CG	EJ	AK	3
	6	AA	CG	BH	KN	3
Family 4						
Parents	**Female**	AC	AH	KQ	GG	
	Male	AC	DD	HH	GL	
Offspring	**1**	AB	CG	EH	IJ	NO MATCH
	2	AC	CG	EH	IK	NO MATCH
	3	AC	CC	EI	KK	NO MATCH
	4	AC	CG	EI	IJ	NO MATCH
	5	AB	DG	EH	KK	NO MATCH
	6	AB	CG	IQ	KK	NO MATCH

3.4.4.4. Polymorphism and Heterozygosity

Table 7 indicates that there was little variation in numbers of alleles between the three groups of fish. The 1997 parents do have fewer alleles at Ssa197, however there was an increase in allele number at Ssa171 compared to the other groups. Locus Ssa197 was found to be the most polymorphic in all groups with 15-19 alleles, locus Ssa85 was also very polymorphic with 14-15 alleles.

Table 3.7: Number of alleles in the 1996 and 1997 parents and Skerries offspring.

Stocks	**Locus**			
	Ssa202	**Ssa171**	**Ssa197**	**Ssa85**
1996 Parents	9	10	19	15
Skerries Offspring	9	10	16	14
1997 Parents	10	12	15	14
Total no. of different alleles	10	13	19	16

Heterozygosity at loci Ssa202, Ssa197 and Ssa85 were very similar in the 1996 parents and the Skerries stocks (Table 3.8). However heterozygosity (Ho) at locus Ssa171 is markedly different, with the incidence 12 % lower in the parental stock compared to the Skerries stock. The 1996 parents appear to be more variable in terms of Ho than the 1997 parents at two loci, Ssa202 and Ssa85. However, at Ssa171 and Ssa197 Ho was lower by 3 or 4 % in the 1996 parents than the 1997. Observed heterozygosities in the 1996 parental stock differed from the expected values at Ssa202, Ssa171 and Ssa197 by 7, 10 and 6 % respectively, the values at Ssa85 only differed by 2 %. Table 3.8 shows an excess of heterozygotes at Ssa202, all other loci were found to be deficient in

heterozygotes to varying extents. Ssa171 was the most deficient by 11 %. In contrast observed heterozygosities were only slightly different than expected in the Skerries stock, with Ssa202 exactly the same. Expected heterozygosity in the 1997 parents was very similar to the observed for loci Ssa197 and Ssa85, Ssa202 was found to have more heterozygotes than would be expected, however Ssa171 was found to be deficient. In comparing the different groups it can be seen that both year classes of parents are very deficient in heterozygotes at Ssa171. Comparison between Ho and He within year classes 1996 and 1997 gave no significant differences using two tailed t-tests, comparison of Ho between year classes also gave no significant differences.

Table 3.8: Heterozygosity (%) in the 1996 and 1997 parents and Skerries stocks.

Stocks		**Locus**			
		Ssa202	**Ssa171**	**Ssa197**	**Ssa85**
1996 Parents	**Ho**	84.0	70.9	81.1	78.9
	He	76.9	81.8	86.9	81.2
Skerries Offspring	**Ho**	79.4	83.2	84.8	83.2
	He	79.4	81.3	88.5	82.8
1997 Parents	**Ho**	73.8	73.8	85.1	71.3
	He	68.4	80.4	84.7	70.5

Ho = Observed Heterozygosity He = Expected Heterozygosity

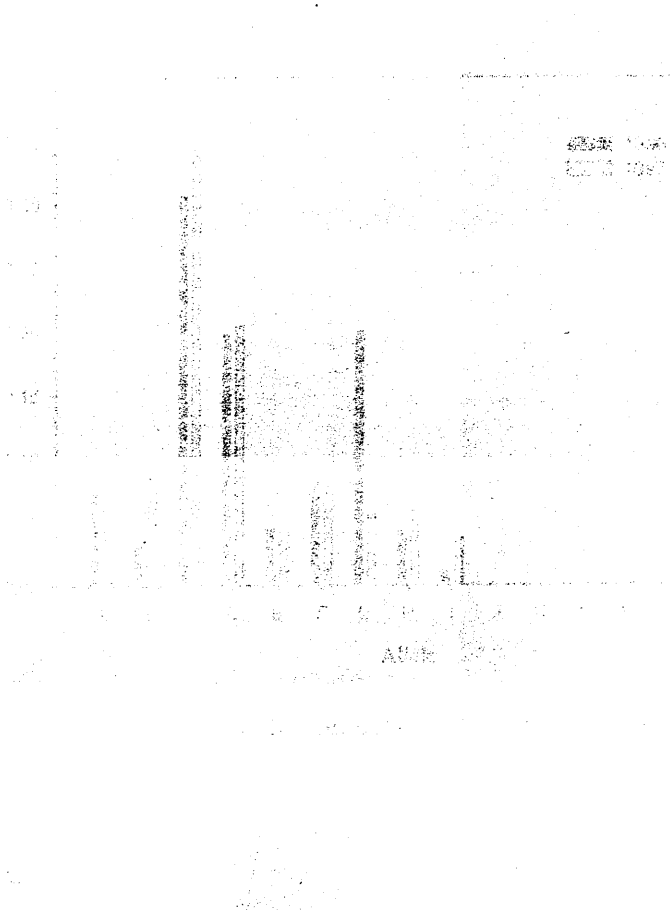
N.B. Expected heterozygosity was computed using Levenes correction.

3.4.4.5. Allele Frequency

Allele frequencies were very similar in each year class at all loci, however larger alleles at loci Ssa202 and Ssa171 were much rarer than smaller alleles (Figure 3.5). Allele A at Ssa202 was very common in both year classes, while allele G at Ssa171 was far more common in the 1997 parents than the 1996. The allele distribution at Ssa197 (Figure

3.6) was quite even, unlike those at Ssa202, Ssa171 and Ssa85; larger alleles at this locus were more common than at others. Allele H at Ssa197 was much more common in the 1997 parents and slightly distorts the even distribution of alleles. Allele distribution at Ssa85 (Figure 3.6) is very uneven, mostly due to allele G which is far more common than any other allele.

Genic differences between populations (year class) were calculated using Genepop, significant differences were found between allele frequencies at each year class at all loci ($p = 0.00000$).



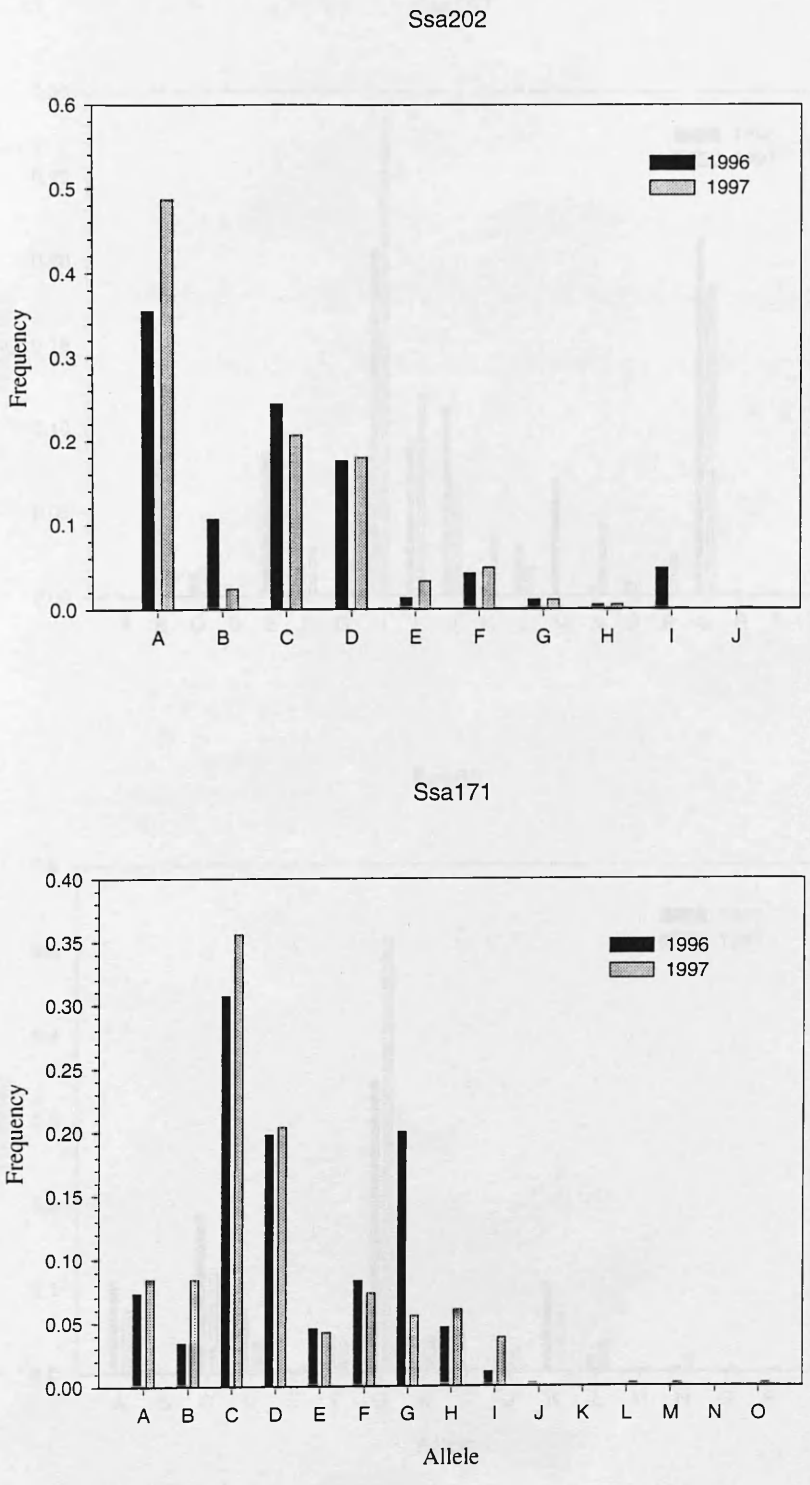


Figure 3.5: A comparison of allele frequencies for each parental year class at loci Ssa202 and Ssa171.

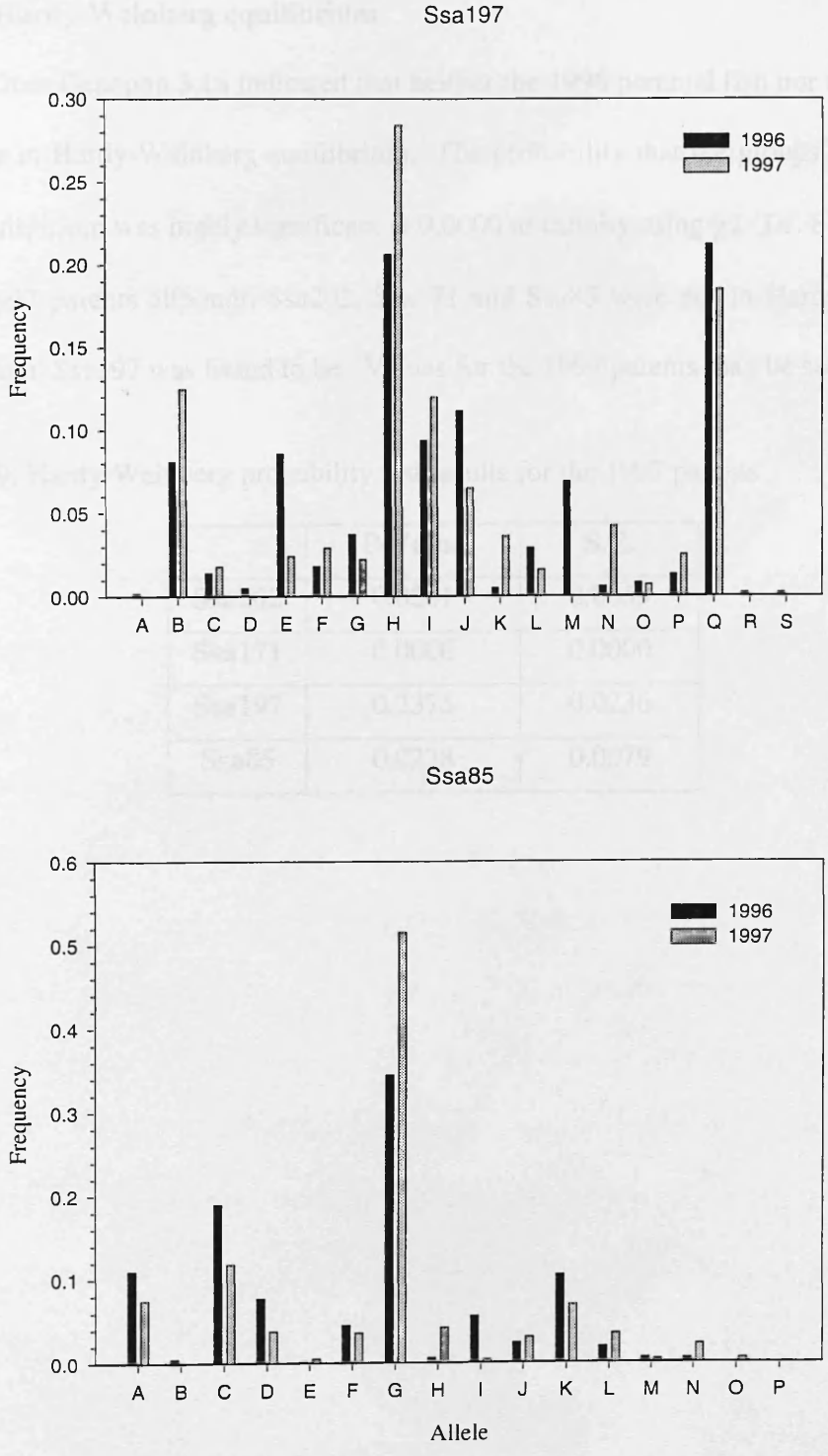


Figure 3.6: A comparison of allele frequencies for each year class at loci Ssa197 and Ssa85.

3.4.4.6. Hardy-Weinberg equilibrium

Results from Genepop 3.1a indicated that neither the 1996 parental fish nor the Skerries fish were in Hardy-Weinberg equilibrium. The probability that the groups were not in H-W equilibrium was highly significant at 0.0000 to infinity using $\chi 2$ (Df: 8). However in the 1997 parents although Ssa202, Ssa171 and Ssa85 were not in Hardy-Weinberg equilibrium, Ssa197 was found to be. Values for the 1997 parents may be seen below.

Table 3.9: Hardy-Weinberg probability test results for the 1997 parents.

	P-Value	S.E.
Ssa202	0.0201	0.0059
Ssa171	0.0000	0.0000
Ssa197	0.2375	0.0236
Ssa85	0.0238	0.0079

3.5. Discussion

The initial task before any genotyping was carried out was to establish that the fish at Out Skerries were suffering from an outbreak of IPN. Bacteria were isolated from the fish and identified as *Vibrio* spp., however they were considered to be opportunistic, non-specific pathogens as determined by the very mixed cultures isolated. IPNV was isolated from moribund fish and histopathology carried out on the Skerries fish was indicative of clinical IPN.

A proportion of the post-smolts sampled at Skerries were very thin suggesting that some of the fish were failing to transfer to seawater and suffering from Failed Smolt Syndrome (FSS). In a classic outbreak of IPN it is often the larger, healthy looking fish which succumb to the disease and although the exact cause of FSS is unknown it has been speculated that IPN may play a contributory role. Smail *et al.* (1995) measured viral titres and histopathology of two groups of Atlantic salmon in Shetland, healthy smolts (mean condition factor, 0.91) and thin failing smolts (mean condition factor, 0.71). Thin smolts demonstrated much higher viral titres than healthy fish, 10^9 $TCID_{50}$ g^{-1} and $10^{4.5}$ $TCID_{50}$ g^{-1} respectively and also exhibited marked pathology unlike that of the healthy fish. The mean condition factor for the fish sampled at Skerries was 0.8, suggesting that the fish were a mixture of both healthy and thin post-smolts. Although IPNV may have contributed to causing FSS in certain cases, possibly indicating a chronic condition, larger healthier fish were also susceptible to the virus suggestive of a classic acute IPN outbreak.

Mortalities during an outbreak of IPN vary from 2% to 20% (Smail *et al.* 1990, Smail *et al.* 1995). From the 4 cages at Skerries that contained the sentinel group mortalities

peaked at 20%, therefore approximately 7800 fish were lost during the outbreak. Only a small number of dead fish were sampled for this study for varying reasons. Although the placement of the sentinel group at Skerries was considered to be the best option at the time the difficulties involved in mortality collection were not fully realised. Skerries Salmon employed divers to collect mortalities once a fortnight, additional dives were required to ensure collection of the majority of susceptible fish. Qualified staff at the farm did carry out an additional dive once during the fortnight increasing collection of mortalities to once a week, but due to time constraints were unable to dive more frequently. This was however still too infrequent to ensure good quality DNA from many fish. Unfortunately due to the location of Out Skerries, an island two hours from the Shetland mainland, there was only one day a week where divers could get on and off the island the same day by ferry. Mortalities decompose very quickly in a farm situation, meaning that many fish were lost before collection distorting the mortality figures. Also those fish that were sampled were only a small proportion of the data set since only whole fish were sampled at each dive, rendering the majority of the mortalities collected unsuitable for DNA sampling.

Analysis carried out on the 1996 parents predicted that 100% of the offspring could be assigned to one or more families and that 83% could be assigned unequivocally to only one family. Once it had been established that the farm was experiencing an outbreak of clinical IPN and that a high proportion of offspring could be assigned unambiguously to one family it seemed reasonable to genotype the DNA samples taken at Skerries. Analysis revealed only one third of the offspring could be assigned to their parents and reasons for this needed to be examined in detail to ensure confidence in the genotyping system. There were four likely possibilities for the lack of assignment; firstly that the

samples had been genotyped incorrectly, secondly that the fish had been mixed at the hatchery with the rest of the production fish whose parents had not been genotyped. A third possibility was that the fish had been mixed during transfer to seawater and upon arrival at Skerries, a fourth possibility was that the given parental crosses might have been incorrectly recorded.

To ensure that each step of the methodology was accurate, the genotyping system was compared to the conventional genotyping system used to type the parents at The Marine Gene Probe Laboratory prior to sampling susceptible fish. A high degree of similarity was established between the two methods and the observed difference in allele sizes was found to be consistent at each locus and between gel runs. Later to support this the Skerries offspring were also genotyped more than once and the same results were obtained each time. A further possibility for the low numbers of individuals assigned to a family was data entry error whilst the data was being transformed for the parentage assignment programme, in an effort to exclude this the processing was repeated in total on 3 occasions with the same results obtained each time.

To examine the suggestion of mixing at the hatchery 64 siblings (one gel run) from the sentinel fish still maintained at the hatchery were genotyped. The number of fish sampled was very small but gave a clear indication that the fish were indeed being mixed at the hatchery as only 64% of the siblings could be assigned to a set of parents. Although this was double the percentage of matches for the Skerries offspring a third of the fish could still not be assigned to their parents. The data suggested that the fish were being progressively mixed firstly at the hatchery and later at seawater transfer and possibly also on arrival at Skerries. It was impossible to check for incorrect parental

crosses, however the increase in matches with fish still at the hatchery suggested that mixing of the fish was more likely to be responsible for the lack of parental assignment rather than incorrect crosses.

In a final effort to prove that the genotyping system and the ABI 377 were accurate a set of four parents from the 1998 sentinel fish and 6 offspring from each pair were genotyped. The DNA was extracted at Landcatch and the PCR carried out at the laboratory at Landcatch and at Stirling. The results indicated that the genotyping system was capable of correctly calling alleles consistently despite the location of the PCR and also that the parentage assignment programme was functioning correctly. Three out of four groups of offspring were correctly assigned to their parents, however the fourth group of offspring did not match the given parents. It was later discovered that the fish had been incorrectly labelled at the hatchery and the wrong set of parents sampled. Yet again the sample size was very small but this only highlighted the fact that a larger sample was not needed to prove that the genotyping was accurate and that there were still problems at the hatchery even in the third year of the breeding programme.

In light of these problems it was decided not to sample IPN susceptible offspring from the 1997 parents. However the genetic data obtained from those fish was still of use in this study to examine variation in terms of allele diversity, heterozygosity and conformance with Hardy-Weinberg equilibrium.

It has been reported that wild stocks of Atlantic salmon are likely to have greater genetic variability than farmed salmon stocks (Cross and King, 1983, Verspoor, 1988).

However upon examination of allelic diversity at the four microsatellites used in this study it would seem that this might not always be the case. O'Reilly *et al.* (1996) reported variation in polymorphism at the four loci in a wild population of salmon in the LaHave River to vary from 9 to 22 alleles. Norris, Bradley and Cunningham (1999) examined allelic diversity in wild salmon from various rivers in Ireland and the Numedalslagen River in Norway using the same microsatellites. In the Norwegian river allele number varied from 9 to 18 alleles, in the Burrishoole, in Ireland, allele number varied from 8 to 15 alleles. In comparison the allelic diversity in the present study was found to vary from 9 to 19 alleles suggesting that this farmed stock was no less variable in terms of number of alleles than wild populations. In support of this Norris *et al.* (1999) also examined allelic diversity in farmed stock of Atlantic salmon in Ireland, the number of alleles was found to be between 10 to 17. Ssa171 was found to be extremely variable in the Canadian population studied by O'Reilly *et al.*, (1996) however it was not the most variable locus in the stocks examined in this study. Ssa197 and Ssa85 were found to be the most variable loci in the present study, similar to that of both Irish and Norwegian populations examined by Norris *et al.*, (1999).

O'Reilly *et al.* (1996) examined heterozygosity in wild populations of Atlantic salmon in Canada for the four loci and found it varied from 80 to 91%. Heterozygosity in both Norwegian and Irish wild stocks were found to be between 79 and 92% (Norris *et al.*, 1999). Although farmed stocks did demonstrate lower heterozygosities, 74 to 86% and 35 to 92 % for Irish and Norwegian stocks respectively, the sample size was very small for the Norwegian stock and may have accounted for this. Heterozygosity varied from 71 to 84% in all groups of Atlantic salmon in the present study, although lower than

wild populations it compares favourably with heterozygosities of other farmed salmon stocks using the same microsatellites.

Although the above measures give an indication of variation within and between populations or groups of fish the frequency of the alleles at each locus is also very important. A group of parents may exhibit very high heterozygosity and have high allelic diversity at certain loci. However, if the distribution of alleles at a locus is not even many parents will share common alleles and the utility of the locus for parentage assignment is likely to be limited due to fewer possible genotypes (Letcher and King, 1999). This was demonstrated to a degree in this study, despite no significant differences between mean heterozygosity between year classes there were highly significant differences between allele frequencies. Using simulations Letcher and King (1999) established that with four loci with a normal distribution and at least seven alleles at each locus it should be possible to identify 100% of offspring to a set of parents.

Norris *et al.* (1999) upon examining variability between two farmed Norwegian stocks and one Irish stock noted that one unnamed Norwegian stock (most likely Akevagen) was much less variable in terms of heterozygosity, allele number and allele size variance than the others. The particular stock was in its sixth year of combined family and individual selection, the farm maintained ~200 families of salmon from each generation and approximately 20% of the families were used to provide parents for each successive generation. Norris *et al.* (1999) suggested that family selection reduced the effective population size by maintaining a limited number of families, resulting in a

decrease in genetic variability in terms of allelic diversity which was not detectable from levels of heterozygosity.

Although the loci used in this study exhibited high heterozygosities and numbers of alleles, the distribution of alleles was not particularly even at certain loci, especially Ssa202 where many individuals shared very common alleles. It is probable that in the stock used in this study the four microsatellites may not have been adequately variable. It is suggested that the number of microsatellites used to identify offspring be increased in the future to allow maximal parentage assignment. Norris, Bradley and Cunningham (2000) noted that increasing the number of microsatellites in their system from four to eight resulted in very little extra discrimination because in their population the extra markers were not very variable. For this reason it is also suggested that any new markers added to the genotyping system be screened for variability within the particular population in use prior to addition.

The Landcatch stock was originally 5 strains of Atlantic salmon that were maintained as separate entities within the hatchery, these strains have since 1996 been used in a cross breeding experiment to eventually homogenise the stock as one. It is not likely that all the year classes have yet been homogenised as regards genetic variation, however the numbers of alleles and heterozygosities observed in the two year classes in this study suggests the cross breeding is succeeding in maintaining genetic variation. It will eventually result in all alleles being present in all generations.

The majority of the data analysed was found not to be in Hardy-Weinberg equilibrium, however many of the assumptions that are made when testing for Hardy-Weinberg

equilibrium are not applicable to farmed stocks. Such significant departures from HW equilibrium indicates that one or more of the preconditions for observing HW equilibrium do not hold (Lessios, 1992). On comparison of the observed heterozygosities with expected there seems to be no consistent reason why the data did not fit into HW equilibrium. From the data generated it is possible to see that certain loci exhibited an excess of homozygotes while others exhibited an excess of heterozygotes. An excess of homozygotes at all loci would suggest that null alleles were a reason for the disequilibrium, however since certain loci exhibited an excess of heterozygotes null alleles were not likely to be the cause of the data not fitting into HW equilibrium. The groups of fish used in this study were from a population that was not infinitely large as a wild population would be and a large proportion of the parental population was tested. Testing such a high proportion of the population gives the study a high power to detect disequilibrium with HW and it would be almost expected that the data would not fit into HW equilibrium since it falls so far short of meeting the assumptions made when testing for HW equilibrium.

During the development of the multiplex PCR various problems were encountered with allele scoring at two loci, Ssa197 and Ssa85, scoring errors were very uncommon at Ssa202 and Ssa171. The use of semi-automated technology simplifies the calling of alleles using GeneScan™ software with preset analysis parameters to call the peaks and Genotyper™ software that later extracts the genotypes. However it was still necessary to manually sort through all individuals which Genotyper™ called as homozygotes at Ssa197 and Ssa85. Differential amplification of smaller alleles over larger alleles at these two loci was the reason for almost all size calling errors, in this situation Genotyper™ would call an individual as a homozygote for the smaller allele. The

reason for this was that Genotyper™ was programmed to filter allele peaks that were less than 32% of the size of the highest allele at a locus, in this way stutter peaks were eliminated. Differential amplification at Ssa85 only occurred when the genotype included the smallest allele (123 bases), however as a precaution all homozygotes at this locus were checked manually in GeneScan™. A possible reason for the differential amplification may be DNA template quality, due to the potential for large numbers of DNA samples it was necessary to use a rapid but crude DNA extraction method. DNA extracted in this way is likely to be of a lesser quality than that extracted by traditional phenol/chloroform methods and may have lead to the preferential amplification of the smaller allele.

Locus Ssa197 was found to exhibit partial null alleles, the larger allele would amplify but often very weakly in comparison to the smaller, Genotyper™ would then call the individual a homozygote. The problem was overcome by manually checking all homozygotes at Ssa197 as for Ssa85 in GeneScan™, however this significantly limited the efficiency of the semi-automated system. O'Reilly *et al.* (1998) also encountered this problem with this locus using radioactive genotyping and suggested it may have been due to DNA sequence variation or deletions in regions encompassing the primer sites. Pemberton, Slate, Bancroft, Barrett (1995) and Callen, Thompson, Shen, Philips, Richards, Mulley, Sutherland (1993) previously suggested this possibility for human genotyping systems.

Fishback, Danzmann, Sakamoto and Ferguson (1999) developed an octaplex and hexaplex PCR system for genotyping rainbow trout, they experienced similar problems to those discussed above. One problem was differential amplification of smaller alleles

over larger alleles, they found that increasing the potassium chloride (KCl) from 50mM to 100mM in the PCR alleviated the problem. This may be considered as a possibility for future work. Fishback *et al.*, (1999) also found problems with 'plus A' modification of certain alleles, this is the non-templated adenylation of the 3' end of the amplified sequence by *Taq* polymerase (Brownstein, Carpten and Smith, 1996). Fishback *et al.*, (1999) found that minimising primer concentrations (0.016 to 0.325 μM) and increasing *Taq* polymerase concentration (up to 3 units in a 15μl PCR volume) resulted in consistent 'plus A' modification of alleles for all loci. Plus A modification was not a problem in the current study, after researching the problem PE Applied Biosystems recommended the addition of 30 minutes at 60°C after the final extension of the PCR with no hold at 4°C. 'A' addition is affected by limiting reagents especially in a multiplex PCR, as a result adenines will not be added to all copies of an allele; alleles can therefore appear as a split peak. A rapid drop in temperature from 72°C to 4°C can also cause problems as a proportion of the adenines may drop off with the sudden change in temperature. The addition of 30 minutes at 60°C caused the adenylation of almost all copies, ensuring single peaks. Primer and *Taq* concentrations used in the present study were very low, however it may be beneficial to examine the effect of increased *Taq* and KCL concentrations on the multiplex as tried by Norris *et al.* (1999) to eliminate problems such as differential amplification.

Although this work was unsuccessful in determining differences in resistance to IPN between families of Atlantic salmon it was still possible to analyse the data for variability. The variation in the farmed salmon stock used in this study was found to be very similar to that of other farmed Atlantic salmon. The utility of the microsatellites in this particular stock was examined and found to be good, although it may be useful if

additional microsatellites are added to the multiplex for increased discrimination. In conclusion it must be acknowledged that this work was carried out using salmon hatched during the first year the breeding programme had been in place at Landcatch. The results have demonstrated the need for a trial period to iron out such unforeseen problems before embarking on a detailed research programme. This study does at least serve to demonstrate some of the problems involved in integrating scientific research with large-scale production. However the problems enabled the accuracy of the fluorescently adapted genotyping system to be thoroughly scrutinized ensuring its' utility for parentage assignment in a commercial situation in the future.

4. Investigations into Variation in Disease Resistance between families of Atlantic salmon and Aspects of the Humoral Immune Response to IPNV

4.1. Introduction

Considering the economic problems caused by IPNV in both Shetland, Norway and more recently in Chile it is desirable to develop vaccines against IPNV or ideally disease resistant fish. To achieve this a reproducible experimental challenge model is necessary to determine the efficacy of a potential vaccine and also to establish resistant or susceptible families of fish. Several challenge models with IPNV in Atlantic salmon post-smolts have been published (Strangeland, Hoie, Taksdal, 1996, Taksdal, Ramstad, Strangeland, Dannevig, 1998). However despite the demonstration of mortality, virus isolation and lesions indicating clinical IPN by Strangeland *et al.*, (1996) their method was found not to be reproducible. Taksdal *et al.*, (1998) found that their fish were latent carriers of IPNV and induction of clinical IPN may have been due to a stress mediated recurrence such as that demonstrated by Roberts and McKnight (1976) in rainbow trout. Currently there is no experimental challenge model available for IPNV in post-smolts and vaccination efficacy trials and disease resistance challenges have been based on the ability of fish to clear the virus after challenge (Ellis, 2000).

Challenges with IPNV in young salmonids have often been successful (Frantsi and Savan, 1971, Elahzary, Lagace, Cousineau and Roy, 1976, Silim, Elazhary and Lagace, 1982, McAllister and Owens, 1986, Bootland, Dobos and Stevenson, 1990, Dorson *et al.*, 1992, Shankar and Yamamoto, 1994). Recently there have been reports of successful IPN challenges using families of very small Atlantic salmon fry (0.17 g) in Norway (Taksdal, Strangeland, Dannevig, 1997, Ramstad *et al.*, 1997). Ramstad *et al.* (1997) found varying degrees of susceptibility between different families of Atlantic

salmon fry. These fish were challenged before the onset of immunocompetence and while this does demonstrate the potential for improving disease resistance to IPN it is unknown how this relates to differences in susceptibility at the post-smolt stage.

Various studies on genetic variation in immune response in fish suggest that certain immune parameters may have potential as markers for disease resistance (Balfry *et al.*, 1994, Fevolden, Roed and Gjerde, 1994, Lund *et al.*, 1995a, 1995b, Wiegertjes, Stet and van Muiswinkel, 1995). The use of marker assisted selection for increased disease resistance is a strategy that among other things circumvents the risk of having to challenge the population with the disease agent (Roed *et al.*, 1992). Knowledge of the immune response of Atlantic salmon to IPNV may reveal an immune parameter that is associated with increased disease resistance and may have potential as an immune marker.

4.2. Aims

1. To establish an experimental challenge model for IPNV in post-smolt Atlantic salmon.
2. To challenge different families of Atlantic salmon post-smolts using the model.
3. To examine differences in mortality and virus titre between the different families.
4. To measure various humoral immune parameters in response to IPNV infection between the families to examine possible mechanisms of resistance.

4.3. Methods

4.3.1. Challenge 1

4.3.1.1. Fish Acclimatisation

Atlantic salmon were obtained from Howietoun Fish Farm at Stirling. The fish farm was regularly tested by the Scottish Office for IPNV and the virus had never been isolated, indicating IPNV free status. The 500 S½'s were transported to the CEFAS laboratory, Weymouth, Dorset, in freshwater, the average weight of the fish upon arrival was 39 g. The fish had been vaccinated using the triple vaccine against *Aeromonas salmonicida*, *Vibrio anguilarum* and *Vibrio salmonicida* with oil adjuvant 2 months prior to transport to Weymouth. The fish were placed into a 2 metre tank of freshwater, the water temperature on arrival was 6° and this was gradually increased over 48 hours to 10°C. The fish were left to acclimatise for 4 days in freshwater. After 4 days the water flow was switched to seawater and within 4 hours the fish were in full salinity seawater.

4.3.1.2. Infective Material

The IPNV isolate was originally isolated from Brindister, Shetland in the summer of 1998 at a site where mortalities caused by IPNV were very high and was kindly provided by Dr. David Smail, SERAD Marine Laboratory, Aberdeen. The virus was cultured by pre-formed inoculation of virus onto CHSE-214 cells; the virus was passaged twice through cells, harvested and titrated as in Chapter 2.

4.3.1.3. Challenge

Prior to challenge kidneys were sampled from 10 fish to ensure the fish were IPNV free. Fish were challenged 48 hours after transfer to seawater and held at 10°C throughout

the trial. The fish were split into 4 groups of 120; 75 fish from each group were injected, the other 45 were to act as co-habitants and were not injected but fin clipped for identification purposes. The negative control group was injected intra-peritoneally (IP) with 0.1 ml of HBSS (Gibco) plus 2% FBS (Gibco). The other 3 groups were IP injected with 0.1 ml of 10^6, 10^5, 10^4 $TCID_{50}$/ml IPNV. For the challenge the fish were moved to four 1 metre tanks, with 14 hours daylight. Fish were fed smolt transfer diet at 1.5 % of their body weight on automatic feeders.

4.3.1.4. Disease Sampling

Fish were monitored twice daily for 28 days, mortalities and moribund fish were removed and the kidney sampled for virus isolation. Moribund fish and fish used for sampling were terminally anaesthetised (Chapter 2). At day 14 a kidney sample was taken from 2 fish from the highest dose tank (10^6 $TCID_{50}$/ml) and the control tank. These samples were processed as in Chapter 2 and placed onto CHSE-214 cells, for a rapid diagnosis samples were also tested by Enzyme Linked Immunosorbant Assay (ELISA).

At day 21, 10 fish were sampled from the highest dose tank and the control tank. All organs were sampled for histopathology, kidneys were sampled for virus isolation, processed and placed onto cells as in Chapter 2. Blood samples were taken from the caudal vein; blood was collected using Terumo 2 ml syringes and Terumo 0.5 x 16 mm needles. The blood was left to clot overnight at 4°C and centrifuged at 3500 g for 5 minutes in an MSE Mistral 3000i centrifuge. Serum was removed and pooled for each group of fish and frozen at -70°C until it could be assayed for neutralising antibody to IPNV.

4.3.1.5. ELISA

A flat bottomed 96 well plate (Iwaki) was coated with anti-IPNV γ globulin purified from rabbit polyclonal antiserum supplied by Keith Way at a concentration of 5 µg/ml in bicarbonate buffer (pH 9.6, Appendix 1) and incubated overnight in a waterbath at 22-26°C. After this time the plate was washed using a rinse and one 2 minute wash with PBS-thiomersal (Appendix 1), in a Dynatech MR7000. Each well was blocked with 200 µl 5% fish skin gelatin (FSG, Sigma) in bicarbonate buffer (pH 9.6), and incubated in a water bath at 37°C for 1 hour. The plate was washed as previously with PBS-thiomersal.

The sample to be tested was used neat and diluted to 1:16 using doubling dilutions made in PBS-Tween20 (PBST) with 2.5 % FSG (Appendix 1), both positive and negative controls were diluted 1:20 in PBST plus 2.5 % FSG. Duplicate 100 µl aliquots of each sample and controls were incubated for 30 minutes at 37°C in a water bath. The plate was washed using a rinse followed by 3 x 2 minute washes with PBST. Biotinylated anti-IPNV γ globulin provided by Keith Way was diluted 1:1000 with PBST plus 1.5 % bovine serum albumin (BSA) (Appendix 1). Into each well 100 µl of biotinylated γ globulin was added and incubated for 30 minutes at 37°C and the previous washing step repeated. ExtrAvidin HRPO conjugate (Sigma) was diluted 1:1000 in PBST+BSA and 100 µl added to each well and incubated for 30 minutes at 37°C. The previous washing step was again repeated and the wells finally rinsed with distilled water. The wells were thoroughly drained by tapping the plate on absorbent paper. The substrate indicator solution was prepared by dissolving one tablet of the substrate, 3,3', 5,5', tetramehyl-benzidine dihydrochloride (TMB, Sigma) in 10 ml of phosphate-citrate buffer with

sodium perborate (Sigma). Into each well 100 µl of the indicator was added to plus 2 extra wells to act as blanks. After 5 minutes the reaction was stopped with 25 µl 10 % H_2SO_4 (Sigma). The plate was then read at 450 nm on the Dynatech MR7000.

4.3.2. Challenge 2

4.3.2.1. Fish

Future challenges were carried out at the SERAD Marine Laboratory at Aultbea, Wester Ross. Atlantic salmon were kindly provided by the Marine Laboratory, the fish had been bought in as eggs and on-grown to S1's on site. Atlantic salmon were put to sea 2 days prior to injection with IPNV; the average weight of the fish was 35 g. Fish were maintained in 1 metre tanks at ambient seawater temperature (Appendix 4) with 12 hours daylength.

4.3.2.2. Infective material

In an effort to increase the virulence of IPNV two isolates of the virus were chosen to passage through fish. The first isolate was from the previous challenge (Br); the second was isolated from Out Skerries, Shetland in the summer of 1998 (Os). Fish were injected with IPNV infected kidney homogenate.

4.3.2.3. Passage 1

A group of 50 Atlantic salmon were anaesthetised (Chapter 2), 20 fish were IP injected with 0.1 ml of $10^{3.5}$ $TCID_{50}$/ml IPNV isolate Br, another 20 fish were IP injected with 0.1 ml of $10^{1.5}$ $TCID_{50}$/ml IPNV isolate Os. The average seawater temperature during the challenge was 7.7°C. After 10 days 5 fish from each group were terminally anaesthetised and head kidney sampled from each fish. At 28 days the remaining fish

were terminally anaesthetised and head kidney sampled; kidneys from group Br. were pooled into 4 groups, kidneys from group Os. were pooled into 8 groups. Kidneys were processed and immediately titrated on CHSE-214 cells (Chapter 2).

4.3.2.4. Passage 2

Based on the results of the earlier passage a group of 25 Atlantic salmon were IP injected with 0.1 ml of kidney homogenate from the previous passage, 10^6 $TCID_{50}$/ml IPNV isolate Os. The average seawater temperature during the passage was 9.6°C. At 10 days post infection all fish were terminally anaesthetised (Chapter 2), head kidney was sampled from each fish and pooled into 5 groups of 5. Kidneys were immediately processed (Chapter 2) and diluted with HBSS plus 2% FBS (Gibco) to give the appropriate volume of kidney homogenate for the family challenge. Kidney homogenate was stored at 4°C overnight.

4.3.3. Family Challenge

4.3.3.1. Fish

Various families of Atlantic salmon were maintained from hatching until 6 months of age in separate tanks at Landcatch. At 8 months of age 2250 fish from 30 families were individually tagged with Passive Integrated Transponders (PIT-tags) (Trovan) injected into the abdominal cavity and maintained together in one tank until seawater transfer. The fish were vaccinated using Furogen 2 against *Aeromonas salmonicida* with oil adjuvant prior to seawater transfer. The fish were transferred to Aultbea Marine Laboratory by road in freshwater and immediately placed straight into seawater upon arrival at Aultbea.

Only 24 families could be used in the challenge for practical reasons, fish were randomly picked from the tanks and subsequently anaesthetised (Chapter 2) and scanned for family data. In each family there was a total of 75 fish; 25 were to be a control group and the other 50 were to be split into 2 groups of 25 to act as replicate infected groups. The fish were allocated to 1 metre tanks according to family with 6 families per tank, the time taken to complete this process was 10 days. Families were allocated to tanks on a first scan basis and replicate tanks did not contain the same mix of families (Figure 4.1). The fish were left for 14 days after this but prior to challenge to acclimatise. The fish were an average weight of 100 g and were at a stocking density of approximately 15 kilos/m^3. Fish were fed transfer diet at 1.5 % body weight on automatic feeders with ambient daylight (approximately 16 hours) and ambient seawater temperature (Appendix 4).

Prior to challenge 50 fish were terminally anaesthetised as above; blood samples were taken from the caudal vein of every fish using 5 ml serum vacuettes with serum separator (Greiner). Head kidney was sampled for virus isolation, pooled into groups of 5 and stored at -80°C until processing. A swab was taken from each pool for bacterial isolation, and the kidneys processed as in Chapter 2. As a measure of health of the fish haematocrit was measured in every fish, the results were grouped into 10 groups corresponding to the pools for bacteriology and virology. Blood was taken up into a non-heparinised capillary tube (Kamlab) and sealed with Cristaseal (Alpha Supplies), the tubes were placed into a micro-haematocrit centrifuge and centrifuged at 9,300 g for 10 minutes. Packed cell volume (PCV) was measured using a haematocrit scale. Whole blood was centrifuged using a Hereas Biofuge A at 4000 g for 20 minutes; serum supernatant was removed and stored at -80°C.

4.3.3.2. Challenge

Fish were challenged 4 weeks after arrival at Aultbea. All fish were anaesthetised as in Chapter 2; fish were either IP injected with 0.1 ml HBSS plus 2% FBS (Gibco) or 0.1 ml $10^{3.5}$ $TCID_{50}$/ml IPNV kidney homogenate. All fish were stressed 3 times a week by lowering the water level for 10 minutes. The average seawater temperature during the challenge was 11.8°C (Appendix 4).

4.3.3.3. Disease Sampling

Fish were monitored twice daily for 7 weeks by staff at the laboratory, mortalities were removed and frozen at -20°C until the kidney could be sampled for virus isolation and PIT tag removed. Fish were sampled at week 3; 2 fish from each family in the control group and 3 fish from each infected family and at week 7; 5 fish from each control family and 10 fish from each infected family (where possible). However only 6 families were selected from the week 7 sampling to be processed, 2 families with low IPNV titres, 2 with medium titres and 2 with high titres at the week 3 sampling. Where *Vibrio* lesions were visible a swab was taken and streaked onto TSA plus salt agar and bacteria identified using methods detailed in chapter 2. Water flow in 4 tanks was interrupted 2 days prior to the week 3 sampling and many fish were lost, 2 tanks of these fish were frozen and kidneys later removed from 10 fish per family to help corroborate the week 3 IPNV titres.

Fish were terminally anaesthetised as in Chapter 2. Head kidney, blood and all organs for histopathology were sampled from each fish (Chapter 2); kidneys were frozen at -70°C until processing (Chapter 2). The blood was left to clot overnight at 4°C and

centrifuged at 4000 g for 5 minutes in a Hereas Biofuge A centrifuge; serum supernatant was removed, aliquoted and stored at -70°C. Virus isolated from every tank at each sampling point was confirmed as IPNV A2 (Sp.) strain by neutralisation titration (Chapter 2).

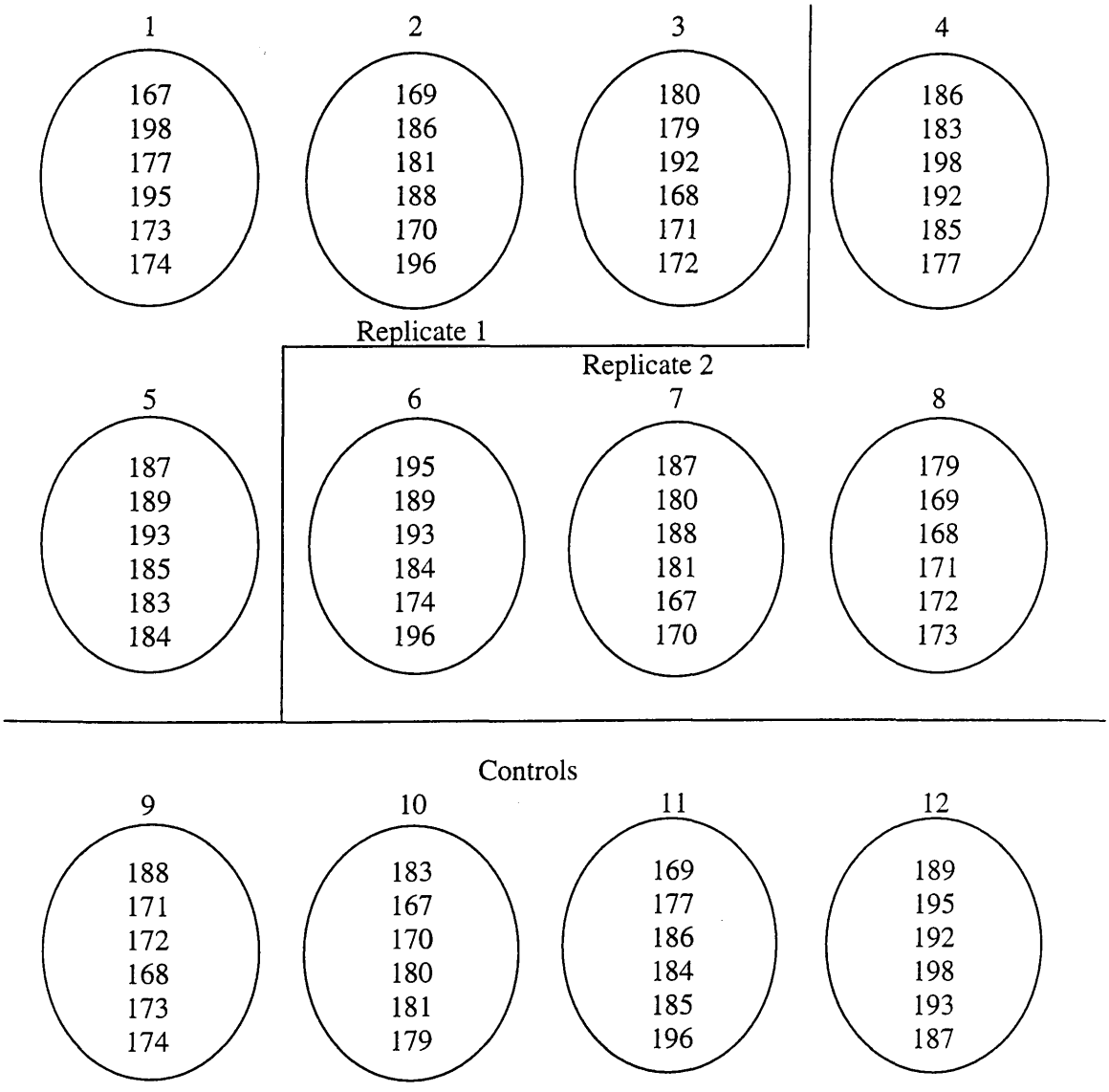


Figure 4.1: Plan of Aultbea Family challenge. Numbers in circles refer to family; numbers outside refer to tank.

4.3.3.4. Additional post-smolt sampling

Post-smolt Atlantic salmon from a different stock to those challenged but still maintained at Aultbea in a different tank room were sampled 4 weeks after the end of the family challenge. Head kidney was sampled from 10 fish, a different scalpel was used for each fish and kidneys freshly processed (Chapter 2).

4.3.4. Immunology assays

4.3.4.1. Neutralising antibody assay

A 1:5 dilution of test serum in EMEM was prepared. EMEM (without L-Glutamine, Gibco) was supplemented with 2% FBS (Gibco), 2 mM L-Glutamine (Gibco), 1x non-essential amino acids (Sigma), 50 IU/ml penicillin, 50 µg/ml streptomycin and 100 µg/ml kanamycin (Gibco). The diluted test serum was heated for 30 minutes at 45°C in a waterbath to inactivate the complement component. Using a flat bottomed 96 well plate (Iwaki) 110 µl of supplemented EMEM was placed into each well. Test serum was diluted 2-fold across the plate beginning with 1:10. Previously titrated IPNV was diluted to give 100 $TCID_{50}$/100 µl and 100 µl added to test wells and mixed. Each plate contained negative controls (no virus) and virus only controls. The plate was incubated at 15°C for 1 hour.

Prior to this a 96 well plate was seeded with CHSE-214 cells and grown to 80% confluence. Each well contained 100 µl of supplemented EMEM plus 10% FBS, using a multi-channel pipette 100 µl of the virus/serum mix and controls was transferred to duplicate wells with the cell monolayer. The plate was incubated in a 2% CO_2 incubator at 15°C until complete CPE was observed in the virus only controls.

4.3.4.2. Lysozyme assay

This assay was adapted from the method of Osserman and Lawler (1966). A 1 % agarose solution was prepared by dissolving 1 g of agarose in 80 ml of sodium phosphate buffer (SPB, Appendix 1). In a further 20 ml of SPB 50 mg of *Micrococcus lysodeikticus* (Sigma) was added and thoroughly mixed by vortexing. Once the temperature of the agar had dropped to 70°C the two solutions were mixed and poured into a bioassay tray (Nunc) to set. Lysozyme standards were prepared using hen egg white lysozyme (HEWL, Sigma). Into 10 ml of SPB 10 mg of HEWL was dissolved to give an activity of 506,000 lysozyme units, 1:10 dilutions were made to give further activities of 50,600, 5060, 506, 50.6 and 5.06. Wells of 6 mm in diameter were punched into the agar and 10 µl of neat serum, lysozyme standards and a control of SPB added to separate wells. Each sample was run in duplicate. The plate was incubated for 24 hours at 22°C. Zones of clearance were measured using calipers (Camlab); lysozyme concentration of serum was estimated from a standard curve of known lysozyme concentrations.

4.3.4.3. Interferon production

Interferon production was attempted to act as a positive control for the interferon assay. A 24 well plate (Iwaki) was seeded with rainbow trout gonad (RTG-2) cells and incubated in a CO_2 incubator at 15°C until at 80 % confluence. RTG-2 cells were propagated in EMEM (without L-glutamine, Gibco) supplemented with 1x non-essential amino acids (Gibco), 2 mM L-Glutamine (Gibco), 10 % FBS (Gibco), 50 IU/ml penicillin, 50 µg/ml streptomycin and 100 µg/ml kanamycin (Gibco) was used to. Polyinosinic:polycytidilic acid (poly I:C, Sigma) was used to stimulate interferon production. In 10 ml of supplemented EMEM without FBS 1 mg of poly I:C was

dissolved to give a concentration of 100 µg/ml. This was further diluted to give 50 µg/ml and 75 µg/ml. Media was removed from the RTG-2 monolayer and 1 ml of each of the three concentrations and a mock of supplemented EMEM without FBS were placed onto the cells in quadruplicate. The plate was incubated in a CO_2 incubator at 15°C for 3 hours after which time 100 µl of FBS was added to give a final concentration of 10 % and the plate further incubated for 72 hours. Interferon was harvested and centrifuged at 400 g for 10 minutes and frozen in aliquots at -20°C. Interferon production was repeated using concentrations of 50 µg/ml, 100 µg/ml, 150 µg/ml and 200 µg/ml of poly I:C.

4.3.4.4. Interferon assay

Measurement of interferon (IFN) activity was attempted using the spectrophotometric assay of Secombes (1994). Various cell lines were tried for this assay; CHSE-214 cells, rainbow trout gonad (RTG-2) cells and Atlantic salmon (AS) cells. A 96 well plate was seeded with cells and grown to 80 % confluence in fully supplemented EMEM (Gibco) containing 10 % FBS. Using a 96 well plate 10-fold dilutions of test serum were made in fully supplemented EMEM with 10 % FBS starting with 1:10 to a volume of 100 µl. The medium was removed from the cells and the cells treated with diluted test IFN samples. Media was also removed from mock rows and replaced with fresh EMEM plus 10 % FBS. The plate was incubated at 15°C in a 2 % CO_2 incubator. After 24 hours the samples were removed and the cells washed with EMEM containing 2 % FBS. Previously titrated IPNV was diluted to 100 $TCID_{50}$/100 µl in 2 % FBS EMEM and 200 µl of virus added to all wells including a positive control row of virus only. As a negative control one row was covered with EMEM only. The plate was incubated at 15°C until advanced CPE was evident in the positive control.

4.5. Results

4.5.1. Challenge 1

4.5.1.1. Mortality

Throughout the trial at CEFAS there were very few mortalities. The control fish experienced the highest mortality, with a loss of 4 fish, however these fish were fin-clipped and death was likely to be due to tail fin erosion. The group injected with 10^4 $TCID_{50}$/ml experienced only one mortality 2 days after challenge that was attributed to stress experienced during infection. The group injected with 10^5 $TCID_{50}$/ml suffered no mortalities and the group injected with 10^6 $TCID_{50}$/ml suffered only 2 mortalities at day 20.

Table 4.1: Mortalities during Challenge 1 at CEFAS.

Days post infection	Controls	10^4TCID_{50}/ml	10^5TCID_{50}/ml	10^6TCID_{50}/ml
2	-	1	-	-
6	1	-	-	-
16	1	-	-	-
20	2	-	-	2

4.5.1.2. Disease sampling

4.5.1.2.1. Mortalities

IPNV was not isolated in cell culture from any of the control group mortalities, but was isolated from the mortality in the lowest dose group and those in the highest dose group. Titration of IPNV from the moribund fish and the mortality in the highest dose group gave titres of 10^6 and 10^5 $TCID_{50}$/ml, respectively. Histopathology from the moribund fish was indicative of clinical IPNV.

4.5.1.2.2. Sampled fish

The 10 fish sampled at the beginning of the trial were IPNV negative in cell culture. Those sampled at day 14 post IPNV injection were tested for IPNV using both ELISA and cell culture; all fish sampled were IPNV negative using both methods.

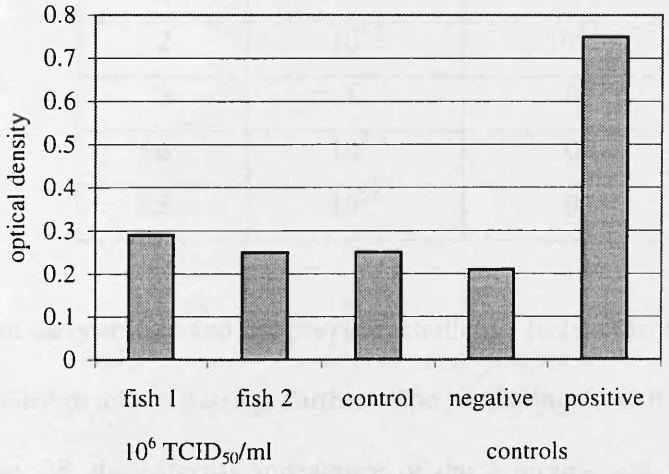


Figure 4.2: Detection of IPNV in the day 14 samples using ELISA.

The fish sampled at day 21 from the control tank were IPNV negative in cell culture. From those sampled from the 10^6 $TCID_{50}$/ml tank only 5/10 were found to be IPNV positive in cell culture. Sera sampled from these fish were found to contain no neutralising antibodies to IPNV.

4.5.2. Challenge 2

4.5.2.1. Passage 1

Mortalities were not produced during this challenge. Table 4.2 shows that at day 10 post infection 4/5 fish injected with isolate Br. (used previously for the challenge at CEFAS) had cleared the virus, only one fish remained infected with a titre of $10^{3.1}$

$TCID_{50}$/g kidney. However 4/5 fish injected with isolate Os. were positive for IPNV with virus titres higher than that injected.

Table 4.2: $TCID_{50}$ IPNV /g kidney at day 10 in a sample of 5 fish for each virus isolate.

Fish	Group Os.	Group Br.
1	$10^{4.1}$	0
2	$10^{4.1}$	$10^{3.1}$
3	0	0
4	$10^{7.6}$	0
5	$10^{5.6}$	0

Based on these early results and the previous challenge isolate Os. was chosen to use in the family challenge and to passage further. The remaining 15 fish in both groups were sampled at day 28, the external appearance of the 2 groups was very different. The group injected with Os. were very dark and thin making them easily distinguishable from the other group. IPNV titres for the group injected with Os. may be seen in Table 4.3. All pools sampled at day 28 from the group injected with isolate Br. were found to be IPNV positive but CPE was visible much later than those from isolate Os. and were not titrated.

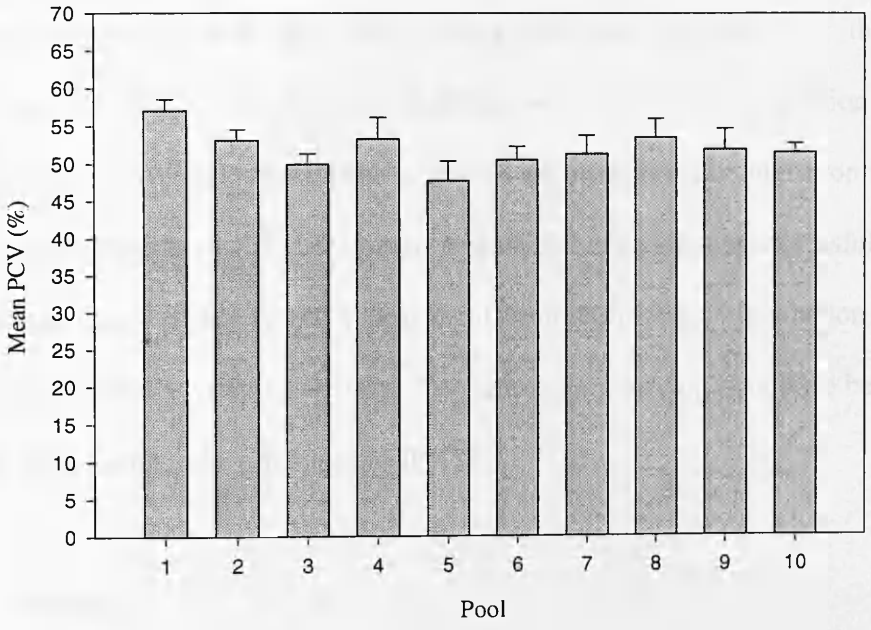


Figure 4.3: Mean (± S.E.) packed cell volume in Atlantic salmon sera (pools of 5 fish) prior to challenge with IPNV.

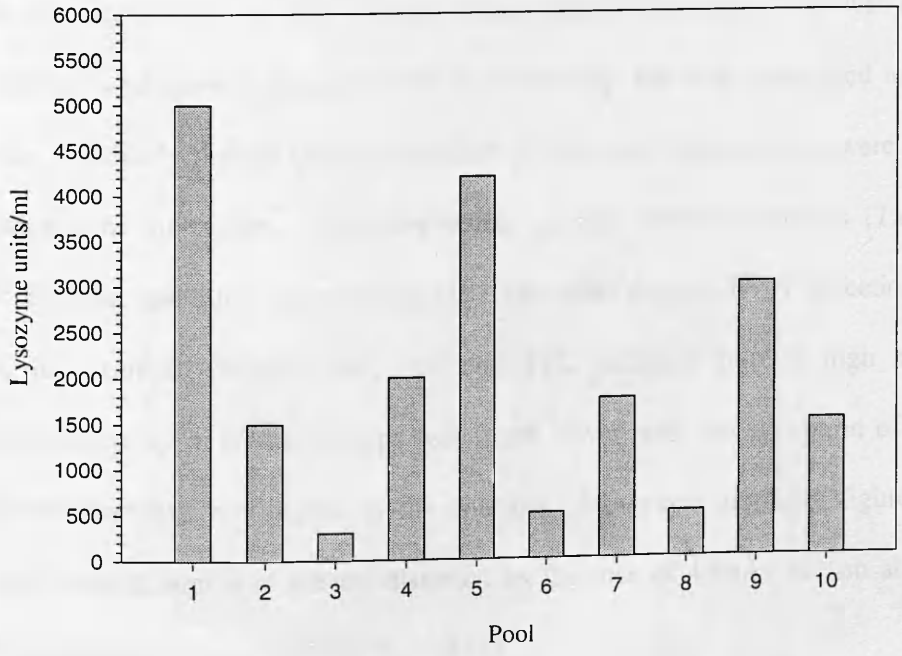


Figure 4.4: Lysozyme activity in Atlantic salmon sera (pools of 5 fish) prior to challenge with IPNV.

Interferon production was apparently unsuccessful as determined by the lack of protection given to RTG-2 cells upon challenge with IPNV after incubation with Poly I:C stimulated RTG-2 cell supernatant. Various attempts to assay interferon in Atlantic salmon serum using both CHSE-214 cells and RTG-2 cells were unsuccessful; both cell lines remained susceptible to IPNV post incubation with serum. Incubation of salmon serum using AS cells was also unsuccessful, although interferon may have been present the cells were found to be refractory to IPNV.

4.5.3.2. Mortality

IPNV was isolated from all mortalities, including all the control fish. Mortality during the challenge was mixed, although some deaths in the infected groups may have been attributable to IPN, the majority of fish are likely to have died of Vibriosis, identifiable by the large lesions on the fish. Where lesions were noted and a swab was taken the resultant bacterial growth was identified as *Vibrio* spp. but was considered to be non-specific. It is likely that all control fish died of Vibriosis, since lesions were noted on the majority of mortalities. Mortality varied greatly between families (Table 4.4), certain families exhibited no mortality (193 and 189) despite IPNV infection, whilst others, for example families 198, 167 and 192, suffered quite a high mortality. Generally mortality in control groups was much lower with the exception of 179 and 168 where mortality was higher in the controls. However, mortality figures in the infected family groups was greatly distorted by the loss of 4 tanks of fish at week 3, therefore no statistical analysis was carried out.

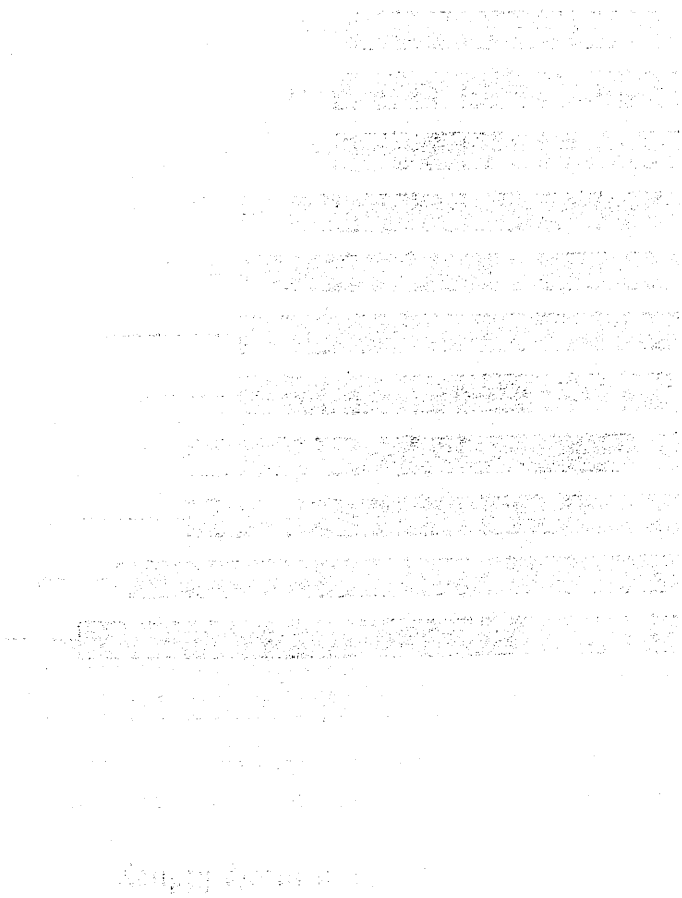
Table 4.4: Total mortality in each family during IPNV infection.

Family	Infected (n=50)	Controls (n=25)
198	18	0
186	13	0
177	13	0
192	16	0
185	5	0
196	9	2
193	0	0
184	1	0
195	4	0
174	7	2
189	0	0
188	12	1
180	6	5
181	4	2
170	14	1
187	5	0
167	16	2
179	1	4
171	1	0
168	3	6
173	4	1
172	1	1
169	11	2

4.5.3.3. Week 3 sampling

4.5.3.3.1. IPNV infected families

IPNV titres in the infected fish varied greatly between families. Figure 4.5 (overleaf) shows that families such as 185, 192, 177 and 189 have very low titres of virus as compared with, 169,188 and 173. The majority of the families have IPNV titres ranging between 10^5 and 10^7 $TCID_{50}$/g kidney. Family 185 has a particularly low virus titre, however 2/3 fish sampled from this family were IPNV negative, 1/3 fish sampled from family 192 was also IPNV negative. No IPN pathology was observed in any of the fish.



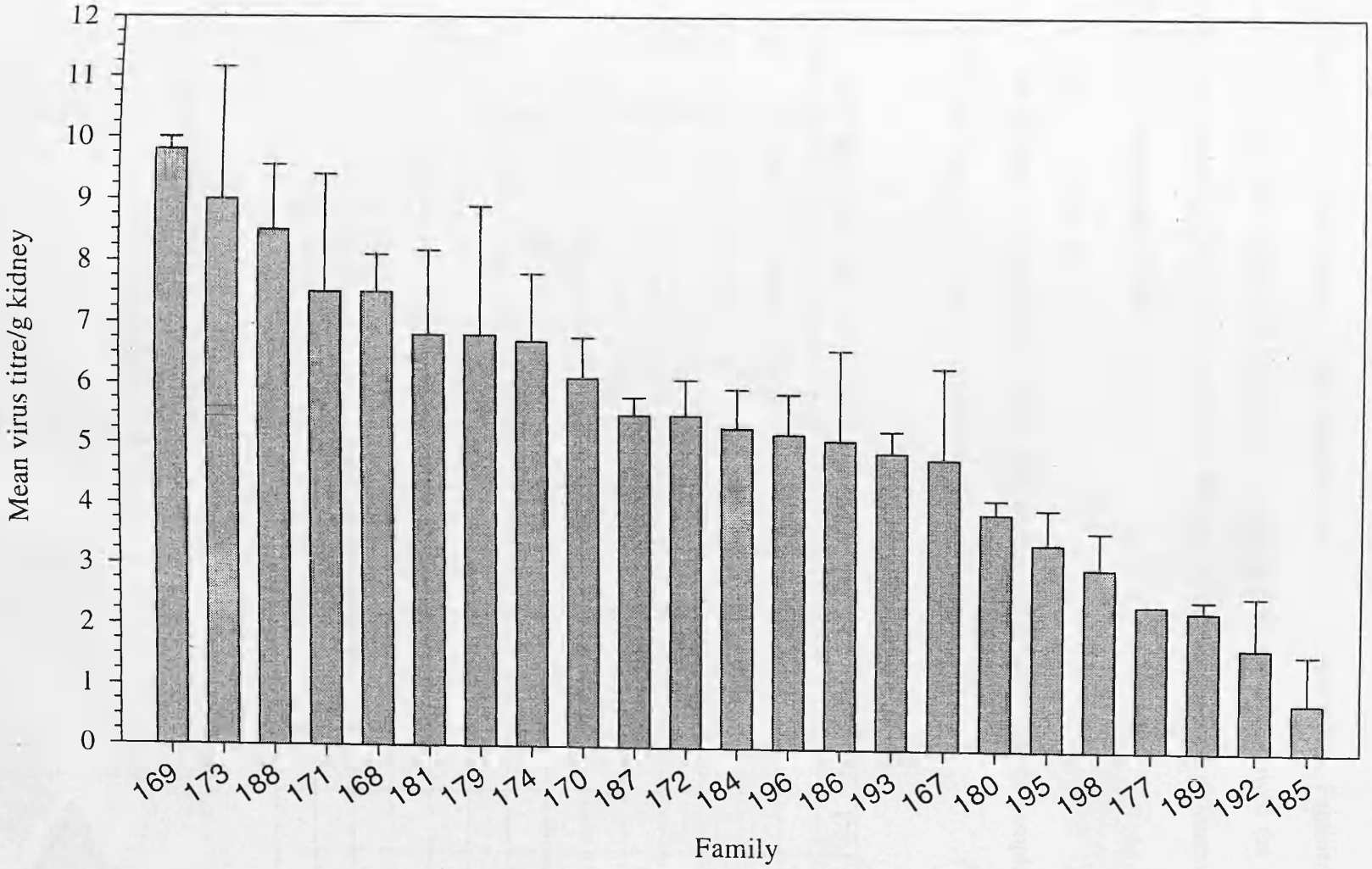


Figure 4.5: Mean (±S.E.) $\log_{10}$ $TCID_{50}$ IPNV/g kidney at week 3 post infection.

A one way ANOVA showed a highly significant difference between the virus titres in each family at week 3 ($F = 4.98$, $p = 0.000$). Tukey's pairwise comparisons showed significant differences between certain families as seen in the table below. Families 169 and 173, those with the highest titres, and 185 with the lowest titre showed the most significant differences with other families. Families with no significant differences at all are not represented in Table 4.5.

Table 4.5: Results of differences in virus titre between families of salmon sampled at week 3 using Tukey's pairwise comparisons.

	177	180	185	189	192	195	198	188	181	169	168	171	173	174
180	-													
185	-	-												
189	-	-	-											
192	-	-	-	-										
195	-	-	-	-	-									
198	-	-	-	-	-	-								
188	+	-	+	+	+	-	-							
181	-	-	+	-	-	-	-	-						
169	+	+	+	+	+	+	+	-	-					
168	-	-	+	-	+	-	-	-	-	-				
171	-	-	+	-	+	-	-	-	-	-	-			
173	+	-	+	+	+	-	+	-	-	-	-	-		
174	-	-	+	-	-	-	-	-	-	-	-	-	-	
179	-	-	+	-	-	-	-	-	-	-	-	-	-	-

N.B. + represents a significant difference.

4.5.3.3.2. Supplementary week 3 samples

IPNV titres in the supplementary fish were very similar to the 3 fish sampled at week 3 in 7 out of the 9 families (Figure 4.6). The two different sampling methods (freezing whole fish compared to immediate sampling) gave very similar results for almost all families, with two main exceptions, family 188 and 169. These families have a lower virus titre in the supplementary fish than the week 3 samples. Family 169 showed a difference of 2 orders of magnitude, but the difference in family 188 was most marked with a difference of 10^3 $TCID_{50}$/g kidney.

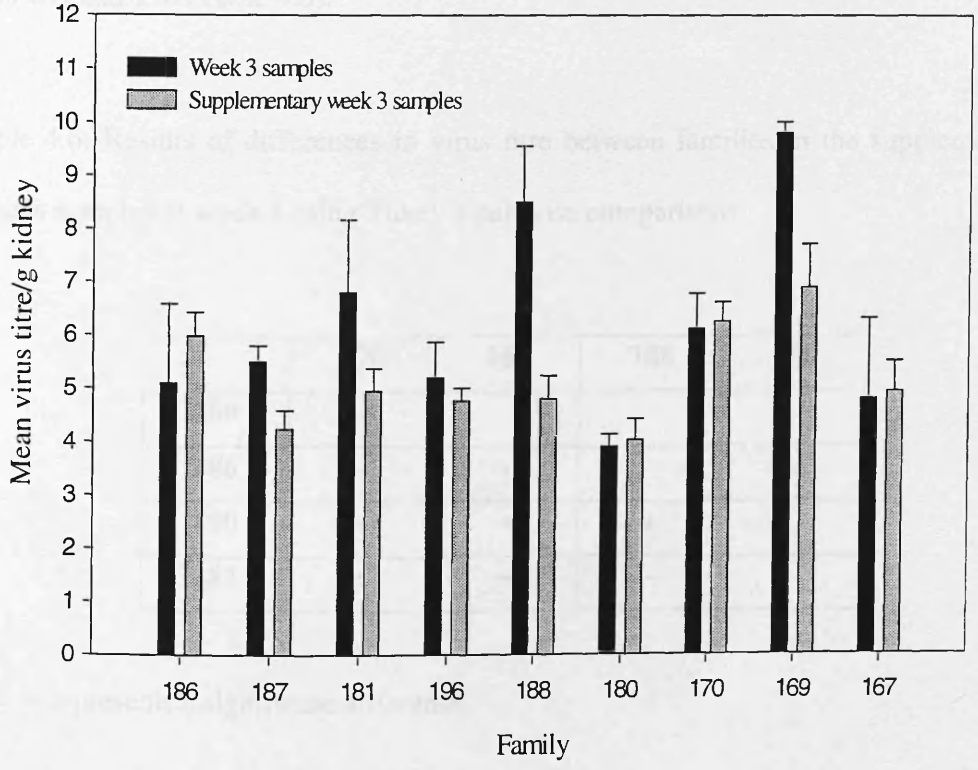


Figure 4.6: Comparison of mean (±S.E.) log_{10} $TCID_{50}$ IPNV /g kidney between the week 3 samples and the supplementary week 3 samples.

A two way ANOVA comparing virus titres between family showed highly significant differences ($F = 7.02$, $p = 0.000$), comparing titre between the two samples but within

family also showed overall highly significant differences ($F = 3.53$, $p = 0.001$) but this was due to one family. However, Tukey's pairwise comparisons among family showed no significant differences between titres in the two groups for every family with one exception, family 188. The two sets of data for family 188 were found to be significantly different ($p = 0.014$).

A one-way ANOVA comparing the virus titres between families in the supplementary group showed highly significant differences ($F = 4.31$, $p = 0.000$). Tukey's pairwise comparisons showed differences between family 180 with 169, 170 and 186, and 187 with 169 and 170 (Table 4.6).

Table 4.6: Results of differences in virus titre between families in the supplementary groups sampled at week 3 using Tukey's pairwise comparisons.

	170	**169**	**186**	**180**
169	-			
186	-	-		
180	+	+	+	
187	+	+	-	-

N.B. + represents a significant difference.

4.5.3.3.3. Control families

Certain families sampled at week 3 were found to be IPNV positive for A2 serotype by cell culture and neutralisation (Table 4.7). In 9 families both fish sampled were positive, in another 2 families only one fish was positive. Almost 50% of the families sampled were IPNV positive, however histology did not reveal any IPN pathology in any of the control fish.

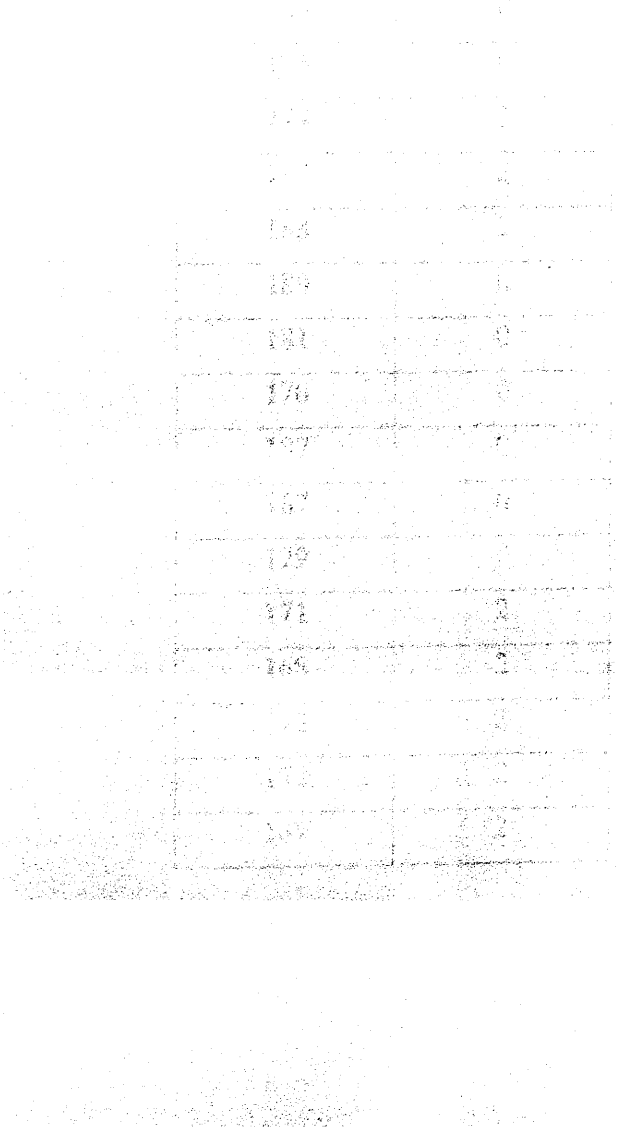


Table 4.7: Number of fish in the control families infected with IPNV at week 3 post injection.

Family	IPNV positive
198	0
186	1
177	0
192	0
185	0
196	2
193	1
184	0
195	0
174	2
189	2
188	2
180	0
181	0
170	0
187	0
167	0
179	0
171	2
168	2
173	2
172	2
169	2

Simple regression was carried out to compare virus titre in infected families with families positive for IPNV at week 3 in the controls (Figure 4.7). Regression showed a significant relationship between titre and control status ($F = 8.92$, $p = 0.007$), although r^2 was only 29.8%.

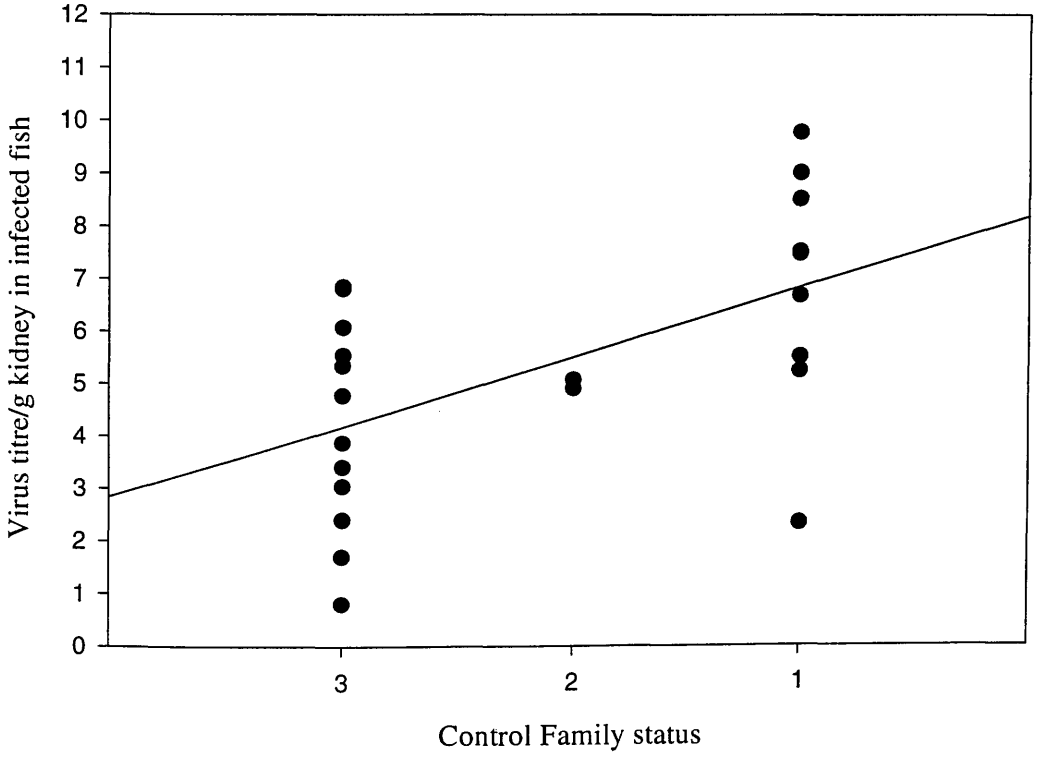


Figure 4.7: The relationship between number of fish infected in the controls and mean $\log_{10}$ $TCID_{50}$ IPNV /g kidney at week 3. N.B. 1 = 2 positive fish, 2 = 1 positive fish, 3 = no positive fish.

4.5.3.3.4. Lysozyme

Lysozyme levels at week 3 were very variable (Figure 4.8), not only between families but also between control and IPN infected groups within families. Families 180 and 169 exhibited low lysozyme in both the controls and infected fish, 2000 units/ml and 4000 units/ml respectively. Whilst families 185, 184 and 187 had much higher lysozyme in the infected fish than the controls, for example in families 184 and 187 the infected fish have lysozyme almost 18,000 units/ml higher than the controls. Families 173 and 169 exhibited very similar lysozyme levels in both the controls and the IPN infected group.

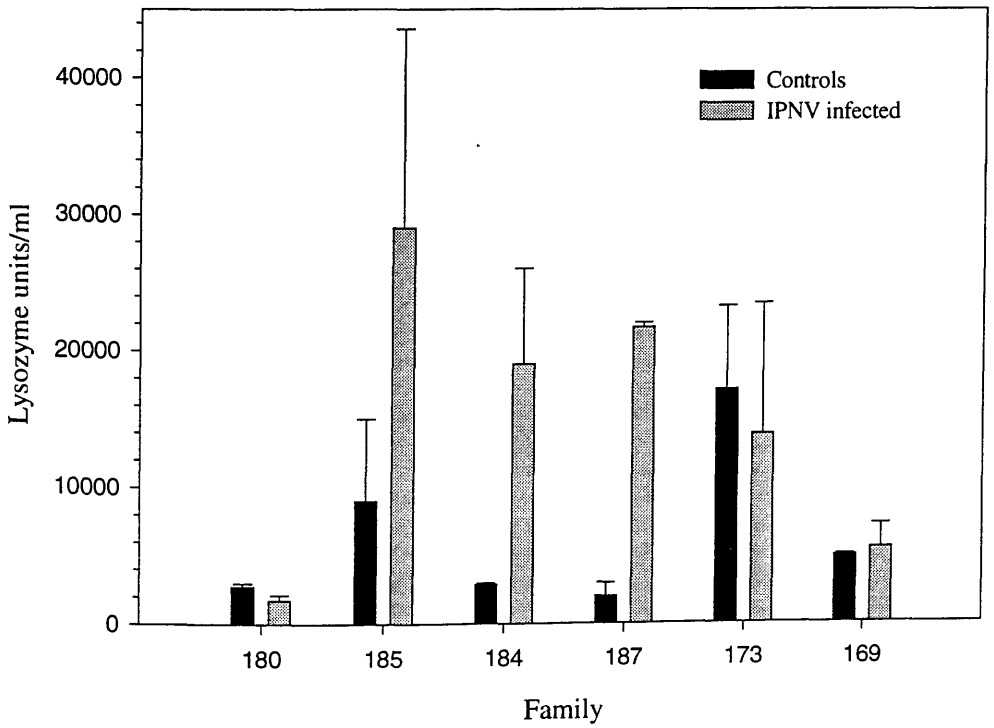


Figure 4.8: A comparison of mean (±S.E.) serum lysozyme activity at week 3 between the control and IPN infected fish.

A two way ANOVA using Tukey's pairwise comparisons compared lysozyme activity between families and also within family but between control and IPNV infected groups at week 3. This showed no significant differences between either families ($p = 0.245$, $F = 1.48$) or control and IPNV infected groups within the same family ($p = 0.189$, $F = 1.66$).

Regression was carried out to compare IPNV titre and lysozyme levels at week 3 (Figure 4.9), the regression showed there was little relationship, $r^2 = 13.2\%$ which was not significant ($p = 0.139$, $F = 2.43$).

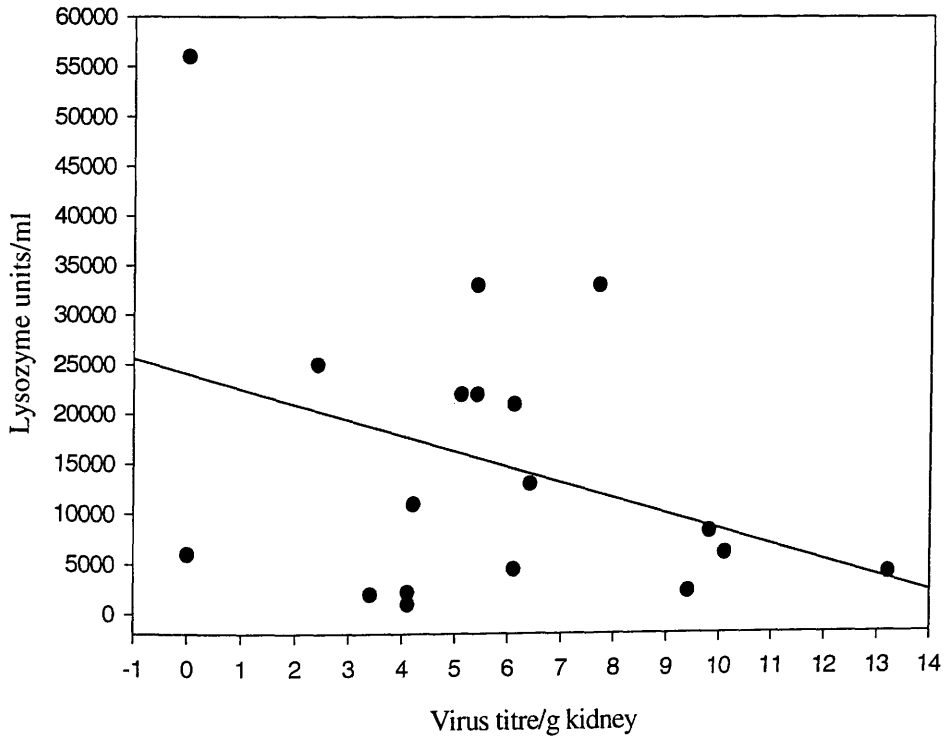


Figure 4.9: The relationship between $\log_{10}$ IPNV $TCID_{50}$/g kidney and lysozyme units in individual fish at week 3.

4.5.3.3.5. Neutralising Antibody

Neutralising antibody titre was generally very low at week 3 (Figure 4.10), with the notable exception of the IPNV infected group of family 185. Families 169 and 173 (with high virus titres at week 3) exhibited no neutralising antibodies to IPNV; family 180 (with low IPNV titre at week 3) also showed no neutralising antibodies in the IPNV infected group but a small amount in the control group (10). Family 185 had very little neutralising antibody in the control group whilst the IPNV infected group of this family were found to have the highest antibody level (~325), however this group were very variable as demonstrated by the large error bar. Families 184 and 187 also exhibited low IPNV antibody titres.

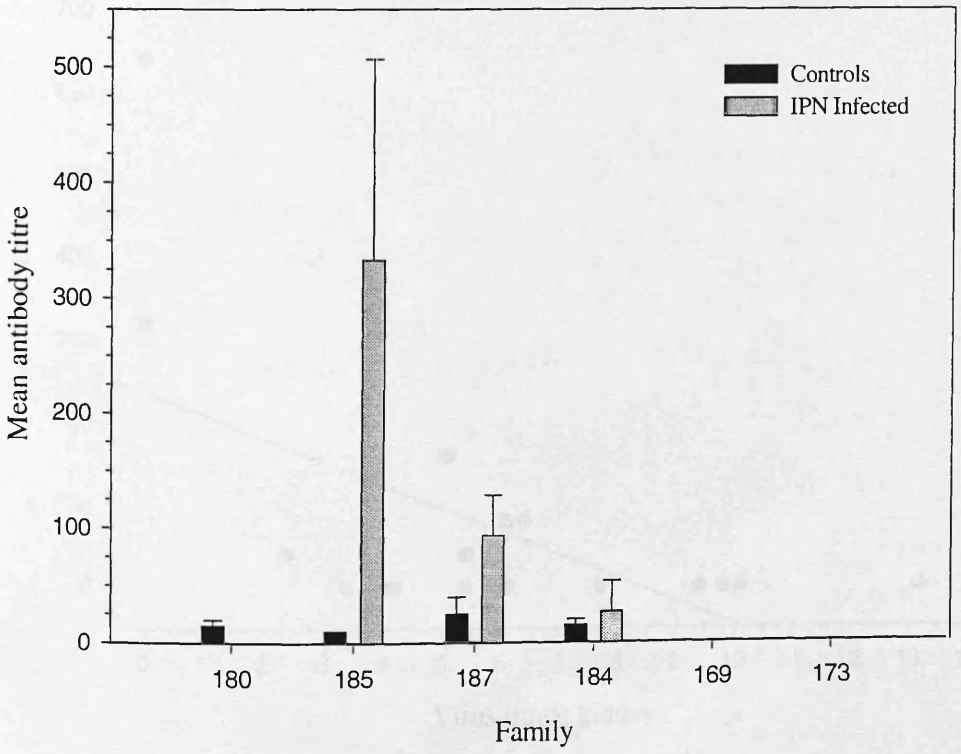


Figure 4.10: A comparison of mean (±S.E.) neutralising antibody titre between controls and IPNV infected fish at week 3.

A two way ANOVA using Tukey's pairwise comparisons compared differences in neutralising antibody between the control and IPNV infected groups and family. A significant difference was found between family ($p = 0.004$, $F = 2.93$) and controls and infected fish ($p = 0.036$, F 5.21). The high antibody titre in the IPNV infected fish of family 185 was found to be significantly different from both groups of family 173 and 169, the infected fish of family 180 and the control fish of family 185.

IPNV titre and antibody titre at week 3 were correlated using regression. Figure 4.11 shows a slight relationship between the two, $r^2 = 35.4$ % which was found to be significant ($p = 0.009$, $F = 8.75$).

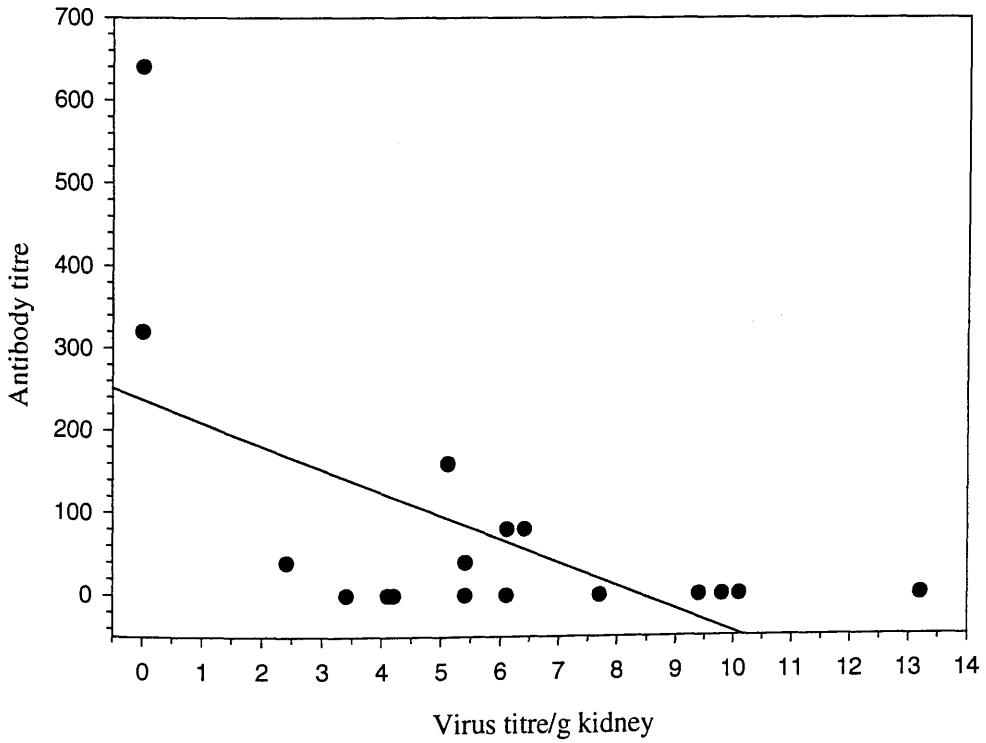


Figure 4.11: The relationship between $\log_{10}$ $TCID_{50}$ IPNV /g kidney and neutralising antibody titre in individual fish at week 3.

4.5.3.4. Week 7 sampling

4.5.3.4.1. IPNV infected families

From the 6 families chosen many of the fish sampled at week 7 had cleared the virus (Table 4.8). Family 187 was the notable exception with every fish sampled still positive for IPNV. In the families with a low IPNV titre at week 3 half the fish sampled were negative, whilst in the medium group there was a complete contrast with one family 100% positive and in the other 44% were negative. In the group with high IPNV titres at week 3 a small percentage of the sample in each family were negative, the majority remained positive.

Table 4.8: Percentage of fish in each family injected with IPNV that have cleared the virus at the week 7 sampling.

Week 3 IPNV titre	Family	% of fish IPNV negative (n)
Low	**180**	66 (9)
	185	50 (10)
Medium	**184**	44 (9)
	187	0 (10)
High	**173**	10 (10)
	169	16 (6)

Figure 4.12 shows that mean IPNV titres at week 7 post challenge were generally very low. The family with the lowest titre was 180 ($10^{0.8}$ $TCID_{50}$ /g kidney), families 185 and 184 were very similar with titres of $10^{1.9}$ and $10^{1.4}$ $TCID_{50}$ /g kidney respectively. Family 187 and 173 had marginally higher IPNV titres of $10^{3.1}$ and $10^{3.7}$ $TCID_{50}$ /g kidney, whilst family 169 had a lower titre of $10^{2.1}$ $TCID_{50}$ /g kidney.

Comparisons of mean week 3 IPNV titres with mean week 7 titres show that in all families except 185 the titre was lower at week 7. Certain families such as 173 and 169 show a large decrease in titre at week 7 by 5 and 7 orders of magnitude respectively. Families 180, 184 and 185 show a reasonable decrease in titre of between 2 and 4 orders of magnitude. However family 185 demonstrates an increase in titre by 1 order of magnitude.

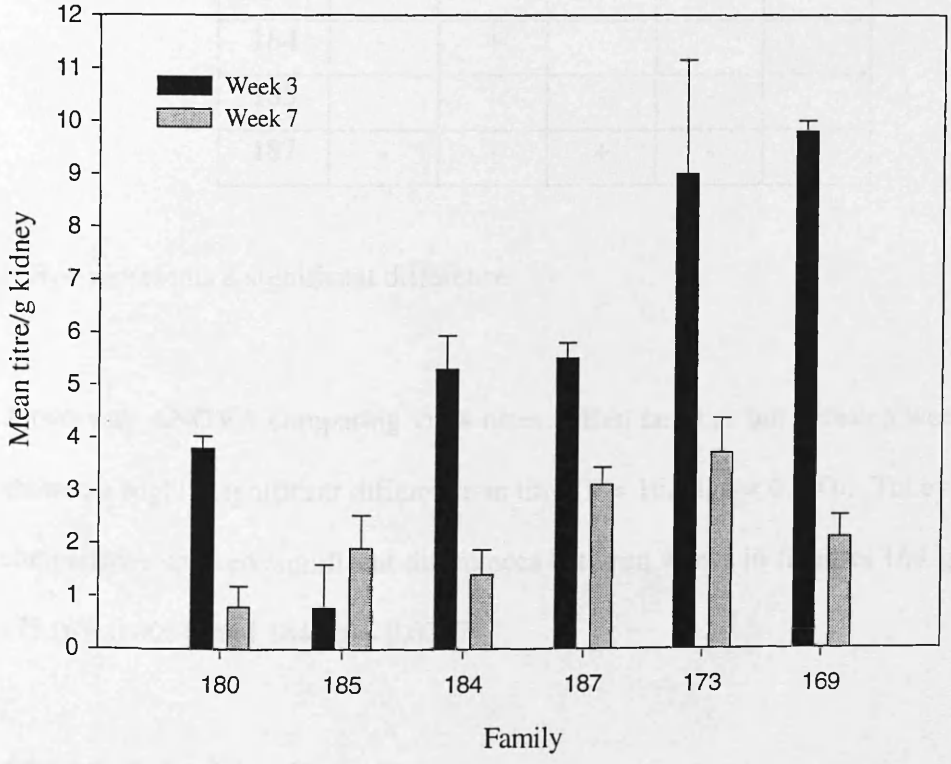


Figure 4.12: Comparison of mean (±S.E.) $\log_{10}$ $TCID_{50}$ IPNV /g kidney between the families sampled at week 3 and week 7.

A one-way ANOVA showed a significant difference between the virus titres in each family at week 7 ($F = 4.50$, $p = 0.002$). Tukey's pairwise comparisons showed significant differences between families 173, 180 and 184 and 180 and 187 (Table 4.9).

Table 4.9: Results of differences in virus titres between families sampled at week 7 using Tukey's pairwise comparisons.

	169	**173**	**180**	**184**	**185**
173	-				
180	-	+			
184	-	+	-		
185	-	-	-	-	
187	-	-	+	-	-

N.B. + represents a significant difference

A two-way ANOVA comparing virus titres within families but between weeks 3 and 7 showed a highly significant difference in titre ($F = 16.55$, $p = 0.000$). Tukey's pairwise comparisons showed significant differences between weeks in families 169 ($p = 0.000$), 173 ($p = 0.0004$) and 184, ($p = 0.0257$).

4.5.3.4.2. Control families

At week 7 every family in the control group was IPNV positive (Table 4.10), confirmed by neutralisation as A2 serotype. In the majority of the families all 5 fish sampled were positive, in 2 families 4 fish were positive and in 5 families 3 fish were positive.

Table 4.10: Number of fish in each family positive for IPNV at week 7 in the controls.

Family	IPNV positive (n = 5)
198	4
186	5
177	5
192	3
185	5
196	5
193	5
184	5
195	3
174	5
189	3
188	5
180	3
181	5
170	5
187	3
167	4
179	5
171	5
168	5
173	5
172	5
169	5

4.5.3.4.3. Lysozyme

Lysozyme levels at week 7 were generally higher than at week 3. Family 180 was unusual in that lysozyme levels were consistently lower than the other 5 families. Lysozyme levels were variable as with the week 3 data. Families 180, 185 and 173 exhibited lower lysozyme levels in the controls than the IPNV infected fish, while families 184, 187 and 169 exhibited the opposite.

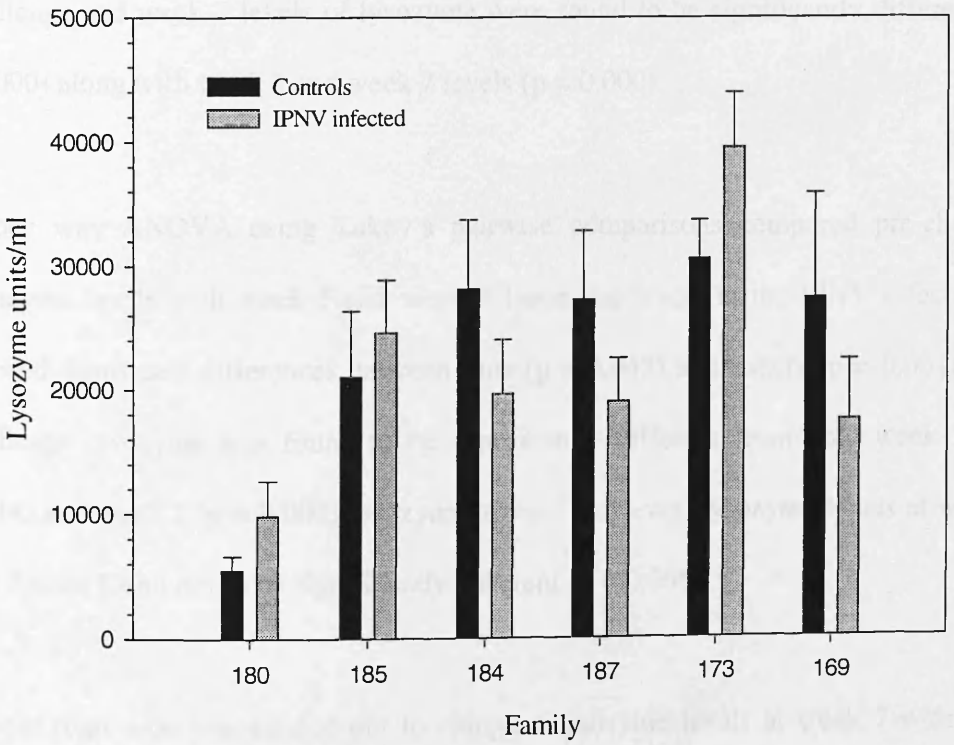


Figure 4.13: A comparison of mean (±S.E.) serum lysozyme activity at week 7 in the control and IPN infected fish.

A two way ANOVA using Tukey's pairwise comparisons compared differences in lysozyme activity between families and between control and IPN infected groups showed a highly significant difference ($p = 0.000$, $F = 6.82$) between families. This was

due to the control group and the IPNV infected group of family 180 exhibiting significant differences ($p = 0.0002$ and $p = 0.0001$ respectively) from the IPNV infected group of family 173. The comparison between control groups and IPNV infected fish within families showed no significant differences ($p = 0.289$, $F = 1.25$).

A one way ANOVA using Tukey's pairwise comparisons compared pre-challenge lysozyme levels with week 3 and week 7 lysozyme levels in the control fish showed significant differences between time ($p = 0.000$) but not family ($p = 0.053$). Pre-challenge and week 7 levels of lysozyme were found to be significantly different ($p = 0.0000$) along with week 3 and week 7 levels ($p = 0.000$).

A one way ANOVA using Tukey's pairwise comparisons compared pre-challenge lysozyme levels with week 3 and week 7 lysozyme levels in the IPNV infected fish showed significant differences between time ($p = 0.043$) and family ($p = 0.001$). Pre-challenge lysozyme was found to be significantly different from both week 3 ($p = 0.014$) and week 7 ($p = 0.000$) lysozyme levels. However, lysozyme levels at weeks 3 and 7 were found not to be significantly different ($p = 0.066$).

Simple regression was carried out to compare lysozyme levels at week 7 with IPNV titre at week 7, little relationship was found (Figure 4.14), $r^2 = 11.6$ % despite this it was still significant ($p = 0.013$, $F = 6.67$).

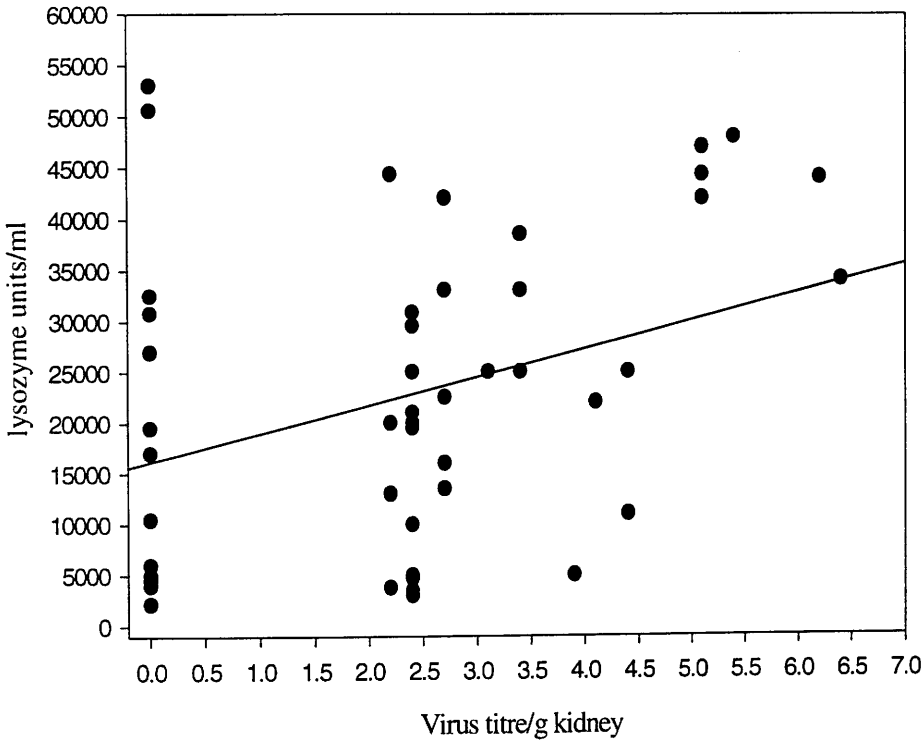


Figure 4.14: The relationship between $\log_{10}$ IPNV $TCID_{50}$/g kidney and lysozyme units /ml in individual fish at week 7.

4.5.3.4.4. Neutralising Antibody

All families showed a higher titre of neutralising antibody at week 7 than week 3. Generally at week 7 the IPNV infected fish showed a higher titre of neutralising antibody than the control fish (Figure 4.15), with the exception of family 169 which was the reverse. The control fish in families 180, 185, 184 and 173 all exhibited very low antibody titres (<500), whilst titres in families 187 and 169 were higher (1200 and 2000 respectively). Families 180 and 169 in the IPNV infected fish have low levels of antibody (600 and 400 respectively), whilst the other 4 families have higher levels ranging from 1100 to 3600. As can be seen from the large error bars neutralising antibody is subject to large individual variation.

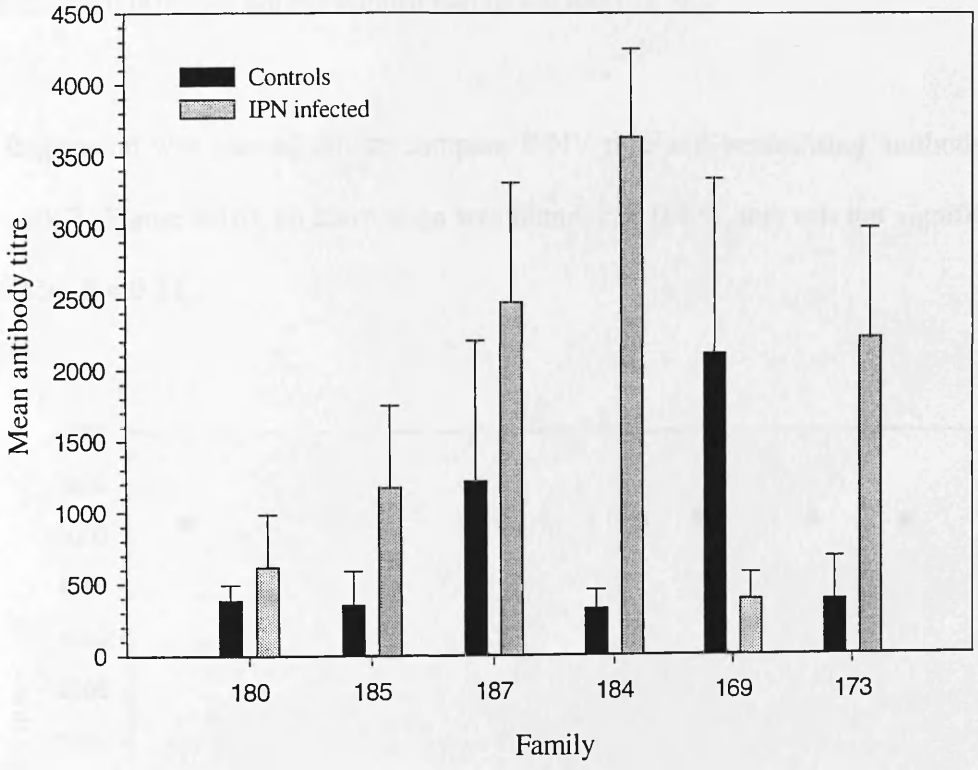


Figure 4.15: A comparison of mean (±S.E.) neutralising antibody titre at week 7 between control and IPNV infected fish.

A two way ANOVA using Tukey's pairwise comparisons compared differences between neutralising antibody in different families and between control and IPNV infected fish. A significant difference was found between IPNV infected fish in different families ($p = 0.007$, $F = 3.24$) but not the controls. The IPNV infected group of family 184 were found to be significantly different from the IPNV infected group of family 169 ($p = 0.0357$) and family 180 ($p = 0.0241$).

A one way ANOVA using Tukey's pairwise comparisons compared neutralising antibody levels between the IPNV infected groups at weeks 3 and 7 and the control

groups at weeks 3 and 7. A significant difference was found between the IPNV infected fish ($p = 0.001$) but not the control fish ($p = 0.448$).

Regression was carried out to compare IPNV titre and neutralising antibody titre at week 7 (Figure 4.16), no correlation was found, $r^2 = 0.4$ %, this was not significant ($p = 0.650$, $F = 0.21$).

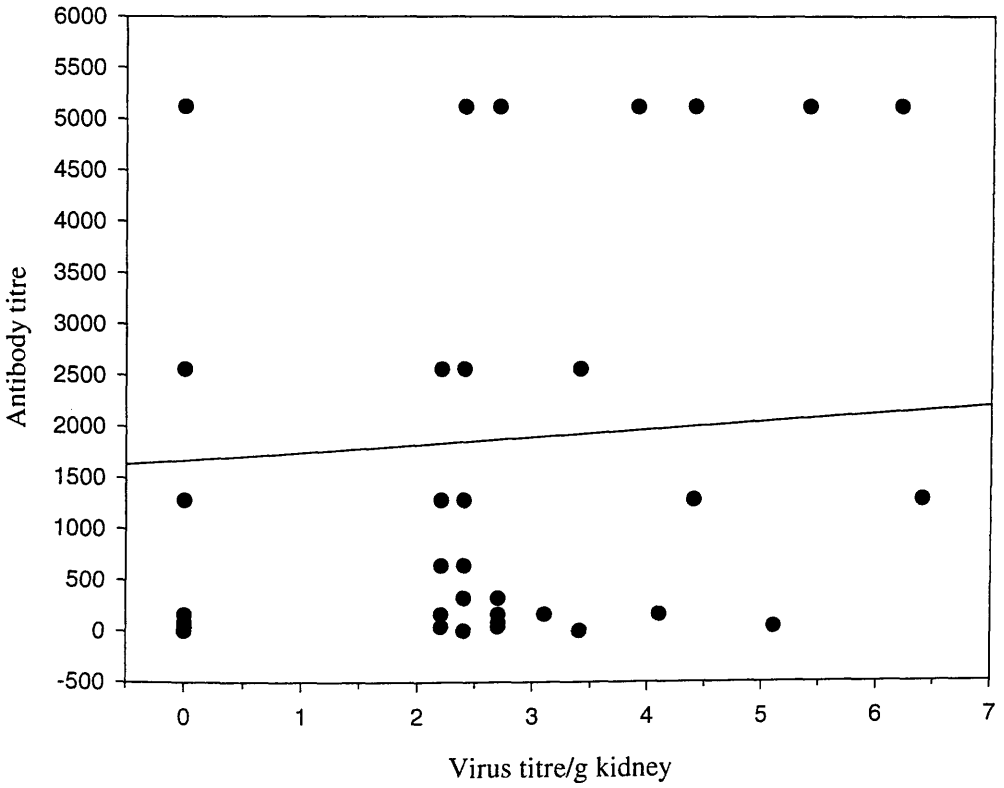


Figure 4.16: The relationship between $\log_{10}$ IPNV $TCID_{50}$ /g kidney and neutralising antibody titre in individual fish at week 7.

4.5.3.5. Additional post-smolt sampling

Virus was isolated from the 8/10 of the additional post-smolts (non-Landcatch fish) sampled and was confirmed as IPNV A2 serotype.

4.6. Discussion

Clinical IPN is very rarely reproduced under experimental conditions in Atlantic salmon (Sadasiv, 1995, Ellis, 2000, Reddington, 2000); more often than not fish become carriers of the virus and lack any attendant disease signs. Indeed clinical IPN was not induced in any challenge carried out in this study for varying reasons.

In considering the challenge at CEFAS there were a few possibilities for the lack of success with the challenge. The Br. isolate used was chosen from a site in Shetland that had experienced a particularly bad outbreak of IPN the previous summer, the isolate was therefore considered to be highly virulent. However, to ensure there was adequate virus available to inject all the fish the isolate was passaged twice through CHSE-214 cells. This may have greatly reduced the virulence of the isolate so that many of the fish were capable of clearing the virus within 2 weeks post challenge and only 50 % remained positive at 3 weeks post challenge. Dorson (1977) demonstrated attenuation of IPNV after only a few passages in cell culture and noted that for experimental transmission of the disease to be successful the virus must have been passaged less than three times in cell culture. Hill and Dixon (1977) reported most of the IPNV strains isolated from rainbow trout in the UK maintained their virulence when passaged less than five times in cell culture.

In constrast Rimstad, Poppe, Evensen and Hyllseth (1991) demonstrated that infecting post-smolt Atlantic salmon with cell cultured IPNV (A2 serotype) caused no clinical signs and no mortality and evidence of the virus could not be demonstrated immunohistochemically. This suggests that the virulence of the virus is altered in some way during cell culture. It is possible that the isolate may have appeared more virulent

than in actuality, the Br. isolate was chosen because of the very high mortalities experienced at the fish farm. However, the practice at the site of collecting IPN mortalities by vacuum pump may have influenced the mortality by disturbing virus and creating an IPNV 'soup'.

The fish used for the CEFAS challenge were from Howietoun Fish Farm. Ideally fish from Landcatch would have been the best fish to use to develop the challenge since they were known to be susceptible to IPN in the field, however fish were unavailable and an alternative source was found. It is possible that different strains of Atlantic salmon may vary in their susceptibility to disease. Fish from Howietoun may have possessed a naturally higher resistance to IPNV and this may also explain the lack of success with the challenge. Variation in intraspecific resistance to disease in various species of salmonids has been well documented. Silim *et al.* (1982) found that on challenging two strains of brook trout with IPNV a strain from Nebraska were more than twice as susceptible than a strain from Quebec. This group found similar differences in susceptibility to IPN for rainbow trout of different origins. Okamoto, Matsumoto, Kato, Tazaki, Tanaka, Hanada, Susuki, Takamatsu, Tayama and Sano (1987) demonstrated differences in susceptibility to IPN between two strains of rainbow trout, one strain suffered almost 100 % mortality, whilst another suffered only 5 % mortality.

The possibility that the fish may have been previously exposed to the virus and may have been latent carriers of IPNV was also considered, to test this sera was tested for neutralising antibodies to IPNV. The sera was found to contain no neutralising antibodies and since all control fish tested were IPNV negative it must be assumed that the fish had not been previously exposed to the virus.

The experience with the challenge at CEFAS suggests that Atlantic salmon were able to mount an effective immune response against cell cultured IPNV that was likely to be non-specific.

To establish if the isolate used in the study at CEFAS had been virulent and to compare virulence with another field isolate a further small scale challenge was carried out at the SERAD laboratory on the north west coast of Scotland. Atlantic salmon from Aultbea were injected with kidney homogenate of two isolates of IPNV, both from Shetland. It became apparent at the 10 day sampling from analysis of virus titre that although neither isolate had caused mortality the isolate used in the previous challenge was not particularly virulent despite the use of kidney homogenate. Indeed 4/5 fish sampled had cleared isolate Br. unlike those injected with isolate Os. External examination of the two groups of fish showed a marked difference in appearance, the group injected with isolate Br. used in the previous challenge appeared perfectly healthy, whilst the other group were dark and thin.

Silim *et al.* (1982) demonstrated variation in virulence of 3 IPNV isolates; one isolate was found to be less virulent than other isolates in both lake trout (*Salvelinus namaycush*) and brook trout. LaPatra, Fryer and Rohovec (1993) upon challenging rainbow trout with different isolates of IHNV found variation in virulence between three isolates of type 3 IHNV, this was thought to be linked to geographical origins of the isolates. In order to increase the virulence of the isolate Os. chosen for the family challenge and to ensure enough kidney homogenate was available to inject all 1200

family fish the isolate was passaged through fish once further prior to use in the challenge.

The pre-challenge samples taken from the family fish indicated that the fish were not only IPNV free but also apparently free of any bacterial infection. A lack of neutralising antibodies to IPNV also suggested the fish had not been previously exposed to IPNV. Analysis of PCV indicated healthy stocks; Sandnes, Lie and Waagboe (1988) found that haematocrit levels ranged between 44-49 % in healthy adult farmed Atlantic salmon. Therefore the haematocrit levels found in this study, ranging between 47-57 % indicated very healthy fish.

Everything possible was considered when setting up the family challenge in an attempt to create a realistic environment, from vaccination to stocking densities. However, clinical IPN was not induced and although mortality was demonstrated it is unlikely that the majority were attributable to IPN despite the isolation of IPNV from all the mortalities. Many of the mortalities displayed external lesions indicative of *Vibrio* spp. infection that were likely to be opportunistic non-specific pathogens as a result of handling stress and seawater transfer. Mortality throughout the family trial was greatly distorted by the loss of many fish from four IPNV injected tanks due to problems with water flow. Although this discounted the possibility of comparing mortality between infected families it was still possible to compare them with the controls. Despite the losses there was a marked difference in mortality between controls and infected groups in many families. Considering all fish were placed under the same amount of stress it is possible that IPN infection may have immunosuppressed certain families, predisposing them to secondary bacterial infections such as Vibriosis.

There have been several reports that IPNV may cause immunosuppression. Knott and Munro (1986) demonstrated a decreased response in Atlantic salmon to phytohaemagglutinin (PHA) in IPNV infected leucocytes compared to uninfected leucocytes. Similar work by Tate, Kodama and Izawa (1990) also suggested that IPNV might have an immunosuppressive effect on rainbow trout T and B cells. This was demonstrated by investigating mitogenic activity of leucocytes infected with IPNV to PHA and lipopolysaccharides; infected cells were found to have a reduced activity. This is consistent with the action of infectious bursal disease virus (IBDV), an avian birnavirus (Chui and Thorsen, 1984). Reasonably then it may follow that virus titre at week 3 may be higher in families with higher mortality. However it appears that there is little relationship between virus titre at week 3 and mortality, certain families with a low titre at week 3 exhibited high mortality and visa versa. This lack of relationship is most likely due to the losses of fish experienced; nevertheless it is possible that in certain families the combination of stress and IPNV infection may have compromised the fish.

Since mortality cannot be clearly demonstrated with IPN another method of identifying susceptible and resistant families of fish was needed. The measurement of viral titre was considered a valid method to estimate susceptibility, although this is an indirect measurement. Virus titre measured in every family at week 3 gave clear and highly statistically significant differences in mean titre for each family. It appeared that although the fish were originally injected with a fairly low dose of IPNV by necessity, the virus was able to replicate and in some families to very high titres. Families with high titres of IPNV were considered to be the most susceptible. Families 169, 173 and

188 in particular had very high titres, but many families had titres of between 10^5-10^7 $TCID_{50}$ /g kidney and may be of intermediate susceptibility. Families 180, 185, 192, 177, 189, 195 and 198 were those with the lowest titres plus significant differences and may be considered to be the most resistant to IPN. It is recommended that further work be carried out using these families since this study can only be considered to give an indication into variations in susceptibility.

These results demonstrated that the different families of Atlantic salmon were not equally susceptible to IPN, but possible reasons for this are unclear. Since all the fish were injected and sampled on the same days and environmental conditions were identical for each group it is likely that the differences are inherent. A very small sample was taken from each family at week 3, this was necessary because of the sheer numbers of fish challenged and to ensure all samples were taken on the same day. The additional samples frozen to back up the results gave very similar titres for all families with the exception of one, and in the supplementary samples families were still significantly different from each other. This suggested that the data sampled at week 3 was reliable. However had more fish been sampled at week 3 a higher number of families would have been significantly different from each other, as demonstrated by the differences found between the supplementary samples at week 3. For example families 169 and 187 were not statistically different at week 3 but were in the supplementary samples despite the almost the same difference in mean titre of 10^3 $TCID_{50}$ /g kidney.

The picture becomes more complicated when considering the incidence of IPNV in the control fish sampled at week 3. Possible reasons for the isolation of IPNV are firstly

that the fish were life long carriers and the stress of transfer, handling and injection and lowering of the water level resulted in a recurrence of the virus as documented by Roberts and McKnight (1976) in rainbow trout. A second possibility was that the virus was present in the seawater surrounding Aultbea Marine Laboratory and the fish were infected naturally.

Traditionally the term 'carrier' was used to describe fish that had survived a clinical outbreak of disease and were apparently healthy but still continued to shed the pathogen (Wolf, Quimby, Carlson and Bullock, 1968). It is widely believed that Scotland does not have the problem of IPNV carrier status such as is evident in Norway where IPN is still a problem in fry and salmon surviving the disease become carriers and take the infection to sea (Jarp *et al.*, 1994). However Norway also has incidences of IPNV in fish with no history of IPN that have been maintained in treated water throughout their time in seawater (Taksdal *et al.*, 1998). Similarly to the present study Taksdal *et al.* (1998) found the kidney samples taken prior to the challenge were negative for IPNV yet still 6 weeks post transfer the control fish were IPNV positive. This suggests that it may be possible that fish do not need to experience an IPN outbreak to become carriers, the virus may be vertically transmitted and persist at very low levels until a time when the fish is stressed and/or a particular set of conditions are met. If IPNV is present in a latent state it may be at levels below the detection limit of current diagnostic methods (Sadasiv, 1995) and this may explain the IPNV negative results.

A further possibility is that the size of sample tested may not be large enough to detect one positive sample, all that is needed to classify a population positive. Thorburn (1996) examined the apparent prevalence of fish pathogens (including IPNV) and its

effect on misclassifying population status. Thorburn stated that a sample of 60 fish from a population assuming a very low prevalence of 2 % gives a 70 % group level confidence assuming 95 % confidence in the diagnostic test sensitivity. The risk of incorrectly classifying a population is 1 minus the group confidence level therefore there is a 30 % chance that 60 samples would not include any test positive fish. The study found that Atlantic salmon were most likely to have a very low apparent prevalence of IPNV and to achieve an acceptable level of confidence it is likely that more than 60 fish should be sampled. Thorburn stated that 149 fish would need to be sampled for any pathogen to achieve a group level confidence of 95 %, unfortunately this is rarely practical and very costly. In this study 50 fish were sampled, giving a group level confidence of ~60 %, it is likely that a higher number of fish should have been sampled and future work should acknowledge this.

The fish may have become infected via the water since the inflow of seawater was not being treated. The presence of salmon farms in the loch where the lab draws its water suggests that horizontal transmission may have occurred, although there were no outbreaks of clinical IPN on any of the farms the fish may still have harboured IPN. The matter of whether the fish were carriers cannot be resolved and the positive status of the stock of fish not from Landcatch suggests that the fish were infected through the water.

The significant although not particularly strong correlation between virus titre at week 3 and IPNV positive status in the controls suggests there may be a slight link between higher titre and positive control status. If the fish were infected through the water naturally the correlation indeed indicates a genetic link, the first families to become

infected were mostly those with higher titres. However if the fish were carriers the titre measured in the infected fish at week 3 may not only reflect the replication of the isolate injected but also replication of virus already present in the fish.

The progress of infection in the two groups, IPNV injected and control fish over the 7 weeks were different. A proportion of the IPNV injected fish had cleared the virus by week 7 or generally had much lower titres of virus. The results showed that although IPNV titre decreased in all but one family by week 7 there were still significant differences between the families. At week 7 the number of fish in each family that had cleared the infection may be more important than titre. The families with the highest percentage of negative fish were the families chosen because of their low titre at week 3, families 180 and 185. Conversely the infection was progressing in the control fish, as shown by the increasing numbers of fish and families infected by week 7. This natural infection still did not cause mortalities, but the difference in progression of infection may indicate that infecting fish by IP injection with a large inoculum of virus is not the most effective method to use. IP injection is often considered not to be the best method of challenge because it bypasses the action of external mucosal immune functions and gut-associated lymphoid tissue (GALT). Since the control fish underwent a natural infection with IPNV and suffered few mortalities it is unlikely that the method of administration of IPNV (IP injection) is the reason for the lack of success with the challenge.

The inability of various research groups and this study to induce clinical IPN has led to speculation that the severe disease outbreaks seen in post-smolts in Shetland and Norway may not be caused by IPNV alone. However other pathogens are rarely

isolated during an IPN outbreak. A further possibility is that there may be certain environmental pre-requisites for the disease that are for some reason not met in an experimental situation. Jarp *et al.*, (1994) suggested that for clinical IPN to occur in post-smolts there must not only be the presence of the virus but also an imbalance of some kind between the environment and feeding causing added stress to the fish.

A Shetland isolate was chosen for all the challenges presented in this chapter for the reason that Shetland experiences particularly bad IPN outbreaks and the isolates may be more virulent than those from mainland Scotland. There was a suggestion that since IPNV was so virulent in Shetland it was possibly a new serotype (Ross and Munro, 1995). Pryde, Melvin and Munro (1993) had previously sequenced a 613 base pair region of the major IPNV structural epitope VP2 (a serotype specific epitope) of a Shetland isolate and the A2 (Sp.) reference strain. They found the two to differ by only 1 % (6 nucleotides) and concluded the Shetland isolate was of the A2 serotype, the deduced amino acid sequences revealed a difference of only one amino acid. However, upon examination of various strains of IHNV Kim, Winton and Leong (1994) found that changes of a single amino acid in key proteins might greatly influence the virulence of the pathogen. In the case of IPNV it is possible that the small changes found in the Shetland isolates may well indicate increased virulence but this is not the only factor contributing to the disease since challenges in Scotland with the isolates does not cause mortality.

Park and Jeong (1996) identified VP3 and VP4 as important immunogens of a Korean isolate of IPNV *in vitro*. They concluded that a portion of the VP3 and VP4 epitope must be projected through VP2 to the surface of the viral particles. They noted that

these epitopes were very labile with storage at -20°C and suggested that a proteolytic protein may have remained on the surface of the virus and degraded them. If VP3 and VP4 are indeed important immunogens and are involved in infection and virus neutralisation the storage of IPNV isolates used for the challenges may also be important.

The type 1 interferon system (IFN α and β) constitutes one of the first and most powerful anti-viral defence mechanisms induced in vertebrates during viral infection (Samuel, 1991). Interferon production and detection were unsuccessful in this study. Initially CHSE-214 and RTG-2 cells were used to attempt to detect IFN in Atlantic salmon serum. The cells did not appear to be sensitive to IFN and since IFNs are species specific it was thought possible that the cell lines were incompatible with the serum. CHSE-214 cells were previously thought to be incapable of producing interferon and were therefore thought to be insensitive to it (Dobos, 1995). However, interferon production has recently been demonstrated in CHSE-214 cells in response to IFN supernatants produced by Atlantic salmon macrophages but not in response to stimulation with poly I:C (Nygaard, Husgard, Sommer, Leong, Robertsen, 2000). RTG-2 cells were known to be susceptible to IFN (Dorson, de Kinkelin and Torchy, 1992) and consequently IFN production was attempted using poly I:C to act as a positive control, however this too was unsuccessful for unknown reasons. AS cells also used in this study are apparently both susceptible to IFN and capable of IFN production (Nygaard *et al.*, 2000), in this study the AS cells were found to be refractory to IPNV and IFN production was unable to be measured. The reasons for this are unclear, the original stock was noted as susceptible to IPNV unfortunately this is no longer the case and due to time constraints an alternative source was not found.

Atlantic salmon are considered to have less lysozyme in comparison to other farmed fish species such as rainbow trout (Grinde, Lie, Poppe and Salte, 1988, Lie, Evensen, Sorensen and Froysadal, 1989). Grinde *et al.* (1988) found lysozyme activity to be approximately 5877 units/ml and 413 units/ml in rainbow trout and Atlantic salmon respectively. These units are for kidney lysozyme, the organ reported to contain the most lysozyme (Lie *et al.*, 1989) and using the turbidimetric assay. Lie *et al.* (1989) reported lysozyme units/g kidney to be 33,500 and 2050 in Atlantic salmon respectively, these results are not comparable to those in this study where serum and units/ml were used. However lysozyme levels in this study were found to be particularly high varying from 300 units to 50,000 units/ml. Of the two methods most commonly used to measure lysozyme activity (turbidimetric and lysoplate assays) the lysoplate assay used in this study has been noted to be the most sensitive of the two (Lie *et al.*, 1989) and this may partially explain the very high levels. Another explanation may be the difference in molecular weight (MW) between hen egg white lysozyme (HEWL) and Atlantic salmon lysozyme. The MW of HEWL is 14.3 kD, the exact size of Atlantic salmon lysozyme is not known but Lie *et al.* (1989) estimated it to be between 12 and 13 kD based on fractionation and gel filtration. Since the lysoplate assay is based on diffusion it is tentatively suggested that this difference in MW may result in a differential rate of diffusion of the two lysozymes through the gel. It is also possible that the presence of *Vibrio* spp. in the system may have enhanced lysozyme activity additionally to the handling stress (Fletcher and White, 1976).

The unusually high lysozyme levels found in this study may indicate the fish were very stressed during the challenge, as suggested by Fevolden, Roed and Gjerde (1994). They

estimated genetic variation in post stress lysozyme activity in Atlantic salmon infected with various bacterial fish pathogens. A negative correlation was found between lysozyme in stressed fish and disease susceptibility, this suggested that enhanced lysozyme activity following exposure to a stressor is not indicative of a more resistant fish but a more disease susceptible organism (Fevolden *et al.*, 1994). It is likely the fish were very stressed during the trial, indeed this was intended since it was considered that stress may induce the fish to succumb to IPNV infection as demonstrated by Roberts and McKnight (1976).

Lysozyme was found to be subject to large individual and family variation as previously noted by Roed *et al.* (1993). For example, family 180 exhibited consistently lower lysozyme at both sampling points compared to the other 5 families. Although there was no difference between lysozyme levels in the controls and IPNV infected fish at either week 3 or 7, there was an effect over time. Pre-challenge lysozyme levels were not significantly different from those in the control fish at week 3, however they were different from those at week 7. The situation in the IPNV infected fish was different, pre-challenge lysozyme levels were significantly different from both week 3 and week 7, yet levels at weeks 3 and 7 were not significantly different. This shows that although there was an increase in lysozyme levels in both groups of fish by week 7 it took much longer to increase in the controls. This may suggest that lysozyme is stimulated upon infection with IPNV or it may indicate that the IPNV infected fish were more susceptible to the stress placed on them. Since lysozyme was found to be suppressed in rainbow trout naturally infected with IPNV (Siwicki, Morand, Klein and Kiczka, 1998) the latter seems the most likely. To clarify this point further work is needed to measure

lysozyme immediately post IPNV injection and regularly throughout the following weeks to establish a pattern.

Siwicki *et al.* (1998) examined the effect of dimerized lysozyme (KLP-602) on non-specific immunity in IPNV infected rainbow trout. Total leucocyte numbers, relative leucocyte count, the phagocytic ability of blood leucocytes, lymphocyte proliferation, myeloperoxidase activity in neutrophils, lysozyme activity and total serum Ig levels were studied. Dimerized lysozyme was found to significantly increase all immune parameters and to decrease IPNV mortality compared to untreated fish, conversely IPNV infection was found to suppress all parameters. This appears to confirm the anti-viral properties of lysozyme. In the present study a positive correlation was found between lysozyme and virus titre at week 7 and although significant the r^2 value was only 11 % suggesting very little relationship between IPNV titre and lysozyme levels. Lysozyme was considered to be unsuitable as a marker for resistance to IPN.

Serum samples taken at week 3 were found to contain neutralising antibodies to IPNV confirming that the fish were exposed to the virus in some way from seawater transfer. It is worth noting that fish confirmed to be IPNV negative at week 3 possessed neutralising antibodies to the virus. Similarly to lysozyme neutralising antibody was found to have large individual and family variation. A significant difference was observed between neutralising antibody at weeks 3 and 7 in the IPNV infected fish but not the controls, suggesting that despite the progressing IPNV infection in the control fish neutralising antibody production was stimulated little. It is possible that with a natural IPNV infection neutralising antibody response is very slow and may not play a significant role in defence against IPNV.

The role of neutralising antibody response to IPNV infection has previously been debated, while certain authors report a correlation between neutralising antibody and virus clearance others report a lack of correlation. Wolf, Quimby and Bradford (1963) found that brook trout with little neutralising antibody shed the most IPNV while those with high neutralising antibody titres shed little virus. Yamamoto (1975) and Mangunwiryo and Agius (1988) also reported a correlation in rainbow trout between a decrease in IPNV isolation and increasing antibody titres. Conversely Reno (1976) reported no correlation in brook trout similarly to Smail and Munro in Atlantic salmon (1985). Biering (1997) examined humoral immune response to IPNV in Atlantic halibut and found that although there appeared to be a correlation between neutralising antibody and virus elimination at the population level, no correlation was found at the individual level.

A significant negative correlation was found in the present study between neutralising antibody and IPNV titre at week 3. However, antibody measured at this time is unlikely to be in response to the injected IPNV but previous exposure to the virus. The complete lack of correlation at week 7 suggests that neutralising antibody cannot be used as a marker for disease resistance to IPNV. Whitton and Oldstone (1996) state that virus specific circulating antibodies are generally not the main protective immune response against viral infections in mammals, cytotoxic T-cells are believed to be the most important in reducing viral replication. However evidence has not yet been presented in teleost fish that cytotoxic T lymphocytes participate in resistance to viral infections (Evans and Jaso-Friedman, 1992, Manning and Nakanishi, 1996). Perhaps future

research should investigate this further. It is possible that complement may play a part in viral defence as suggested by Sakai (1992) and this could be measured also.

In conclusion, a reproducible IPNV challenge model was not developed in this study. The challenge with cell-cultured IPNV indicated that Atlantic salmon were able to rapidly clear the virus possibly through an effective non-specific immune response. Kidney homogenate containing virus was found to induce a carrier state rather than clinical infection, however a difference in susceptibility between families of Atlantic salmon to IPNV was found using virus titre as an indirect measurement of susceptibility. The IPNV positive status of the control fish enabled a comparison between IP injection and a natural infection. The natural infection appeared to mimic the situation in the field progressing slowly over many weeks post seawater transfer rather than the rapid infection usually associated with viruses. The enhancement of lysozyme activity in IPNV injected fish suggests that fish carrying IPNV may be more susceptible to stress than uninfected fish and may indicate activation of phagocytic cell populations. The presence of significantly less neutralising antibody in control fish than infected fish suggests that neutralising antibody may not play a significant role in IPNV infection in the field. The detected genetic variation suggests potential for improvement in disease resistance to IPNV in Atlantic salmon.

5. Diagnostic Techniques for IPNV

5.1. Introduction

The lack of adequate control measures for IPNV and the ability of the virus to persist in a carrier state without causing disease indicates the need for highly sensitive and rapid diagnostic methods (Dixon and Hill, 1983, Lopez-Lastra *et al.,* 1994). Although there are at least two IPN vaccines commercially available in Norway, one is an inactivated IPNV vaccine (Pettersen, 1997), the other a sub-unit vaccine using recombinant VP2 (Frost and Ness, 1997) Norway still suffers huge financial losses due to annual IPN outbreaks (Cripps, 1999). The efficacy of recombinant DNA vaccines against IPNV are presently being evaluated in the UK (Ellis, 2000), however none are yet commercially available and this is hampered at present by the lack of adequate challenge models. The carrier status that occurs after a clinical outbreak of IPN is often detectable from head kidney in cell culture (Johansen and Sommer, 1995). Nevertheless it is possible that fish may harbour IPNV at very low levels and never experience an IPN outbreak suggesting that IPNV may be present at levels below the detection limit of standard tests (Taksdal *et al.*, 1998). This is particularly crucial for laboratory studies involving the assessment of vaccines and disease resistance trials.

A recent survey carried out by Bruneau, Thorburn and Stevensen (1999) estimated the sensitivity and accuracy of diagnostic techniques in use for both IPNV and IHNV in fish health laboratories around the world. The Delphi Panel Method used in the survey involves ascertaining the opinions of a panel of experts through an iterative process, answers were obtained to specific questions, the responses summarised and the summary returned for reassessment. The results indicated that although the experts estimated almost 100 % specificity of the tests in use (cell culture, ELISA, gene probes

and IFAT) the sensitivity was estimated at under 70 % for all tests. This highlights the need for highly sensitive diagnostic tests for many fish pathogens not only IPNV. Various diagnostic methods for IPNV were evaluated in this chapter, factors included the time taken for the assay, the cost, the sensitivity and where possible the specificity. All these methods were compared to cell culture for sensitivity.

5.2. Aims

1. To adapt the RT-PCR using published IPNV primers and establish the sensitivity and specificity in tissue homogenates.
2. To develop an ELISA for IPNV detection and evaluate the sensitivity and specificity of the assay.
3. To establish an immunohistochemical staining procedure for IPNV detection in paraffin embedded tissues.

5.3. Methods

5.3.1. RT-PCR

5.3.1.1. Preparation of RNase free equipment

Glassware was initially soaked in disinfectant (Decon) for a two hours and rinsed thoroughly with nanopure water. Bottles, beakers and cylinders were filled to the top with nanopure water and eppendorfs fully immersed in nanopure water, 0.1% v/v diethyl pyrocarbonate (DEPC, Sigma) was added in the fume cupboard. The DEPC solution was pipetted up and down vigorously to disperse the DEPC and left overnight in the fume cupboard. To remove the DEPC all solutions were autoclaved at 15 lb for 20 minutes in an autoclave vented outside before discarding the water.

5.3.1.2. RNA extraction from cell culture fluids

RNA was extracted using a NucleoSpin Virus RNA extraction kit (Macherey-Nagel). RNase free water was placed into a water bath at 70°C to heat prior to extractions. Then 150 µl of purified virus, tissue culture supernatant or kidney homogenate was placed into a 1.5 ml microcentrifuge tube, 600 µl of buffer RAV1 containing carrier RNA (provided with the kit) was added to the sample, pipetted up and down and vortexed well. The mixture was then incubated at room temperature for 10 minutes. If the solution was turbid after this time the tube was centrifuged at maximum speed (13,000 g) for 1 minute. After this or if the solution was clear 600 µl of absolute ethanol was added to the clear lysis solution and thoroughly mixed by vortexing. From this lysis solution 700 µl was placed into a Nucleospin column in a 2 ml microcentrifuge tube and centrifuged at 6500 g for 1 minute. The flow-through was then discarded and the rest of the sample (about 750 µl) then placed into the spin column and centrifuged at 6500 g for 1 minute. Next 500 µl of buffer RAV3 (provided

with the kit) was placed into the spin column and centrifuged at 6500 g for 30 seconds, this washing step was then repeated. The spin column was then placed into a new sterile (RNase free) microcentrifuge tube and centrifuged for 2-3 minutes at maximum speed (13500 g) to remove the last traces of buffer RAV3. The spin column was placed into another new sterile microcentrifuge tube and 50 µl of pre-heated RNase free water (Promega) added to the column, incubated at room temperature for 2 minutes and centrifuged for 1 minute at 6500 g. The resulting RNA was transferred to a fresh tube and stored at -20°C.

5.3.1.3. RNA extraction from tissue

RNA was initially extracted using a NucleoSpin Virus RNA tissue extraction kit (Macherey-Nagel). Atlantic salmon kidneys were stored frozen at -70°C and in RNAlater (Ambion) at -20°C. Kidneys were spiked with 100 µl of 10^{12} $TCID_{50}$ IPNV and RNA extracted. This method of extraction was found to be unsuitable for use with kidney tissue from either storage method and an alternative method was found using Tri Reagent (Sigma).

Kidney was weighed to give 100 mg, spiked and homogenised in 10x volume of Tri Reagent (1 ml) using disposable pestles (Sigma) in an RNase free 1.5 ml microcentrifuge tube. After homogenisation kidney was centrifuged at 12,000 g for 10 minutes at 4°C to remove high molecular weight DNA and insoluble material. The supernatant was transferred to a fresh RNase free 1.5 ml microcentrifuge tube. To allow complete dissociation of nucleoprotein complexes the supernatant was left to stand at room temperature for 5 minutes, 200 µl of chloroform was then added per ml of Tri Reagent. The sample was vigorously shaken for 15 seconds and allowed to stand

for 15 minutes at room temperature. The sample was then centrifuged at 12,000 g for 15 minutes at 4°C. The colourless upper aqueous phase contained the RNA.

This upper aqueous phase was transferred into a fresh RNase free 1.5 ml microcentrifuge tube and 500 µl of isopropanol (BDH) per ml of original Tri Reagent used was added and mixed. The samples were left to stand for 10 minutes at room temperature and then centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was removed and the resultant RNA pellet washed by addition of 1 ml of 75 % ethanol per ml of original Tri Reagent. The samples were vortexed and centrifuged at 7,500 g for 5 minutes at 4°C. The supernatant was removed and the RNA pellet air dried for 10 minutes, RNA was re-suspended in 100 µl of RNase free water.

5.3.1.4. RT-PCR

IPNV primers were synthesised with reference to two published primer sets (Wang, Wi and Lee, 1997) at MWG. Primers were re-suspended in RNase free water to a concentration of 10 µM and stored frozen in aliquots at -20°C.

Table 5.1: Sequence and location of IPNV primers.

Primer set	VP2 location	Sequence	Product Size
PrD	419-693	5'-CGGAAATACGACATCCAGAGC - 3'	274
		5' – TGGCTCCGTTCATGGACTGG - 3'	
PrF	807-1330	5' – GCCGACATCGTCAACTCCAC - 3'	524
		5' – GACAGGATCATCTTGGCATA - 3'	

RT-PCR was carried out using Pharmacia Biotech Ready to go RT-PCR beads in 0.2 ml tubes. Amplification reactions were carried out in a volume of 50µl, with the RT-PCR beads containing 2.0 units of Taq, 10mM tris-HCl, 60mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, Moloney murine leukemia virus (M-MuLV) reverse transcriptase, RNAguard ribonuclease inhibitor and stabilisers. RT-PCR was optimised by amplification at 12 annealing temperatures between 50-60°C on a Biometra gradient thermal cycler along with varying amounts of template. Once an annealing temperature had been chosen primer concentrations were titrated from 0.5 µM to 0.2 µM and concentrations of 0.2 µM for primer set PrF and 0.5 µM for PrD were chosen.

Prior to RT-PCR the appropriate volume of dsRNA and primers were placed into a sterile RNase free 0.2 ml PCR tube (AB Gene) and the dsRNA denatured for 10 minutes at 99.9°C in a PE Applied Biosystems GeneAmp® 2400 thermal cycler, then immediately placed on ice for 2 minutes. Meanwhile the appropriate volume of RNase free water was added to each RT-PCR bead and left for 5 minutes on ice to re-suspend. The denatured RNA and primers were then added to the dissolved RT-PCR bead and RT-PCR carried out in the PE Applied Biosystems GeneAmp® 2400 thermal cycler.

Thermal cycling conditions were:

45 minutes at 42°C (reverse transcription)

5 minutes at 95°C (denaturing to destroy any remaining M-MuLV)

35 cycles of: 1 minute at 94°C (denaturing)

1 minute at 55°C (annealing)

1 minute at 72°C (extension)

10 minutes at 72°C (final extension)

4°C hold

The annealing temperature was increased to 60°C for RT-PCR and the cycles increased to 40 for RNA extracted from tissue.

5.3.1.5. Electrophoresis

RT-PCR products were visualised on a 1 % agarose gel, 1 g of agarose (Sigma) was dissolved in 1x TBE buffer (Appendix 1) by microwave heating. Once the molten agar had cooled to 70°C ethidium bromide (Sigma) was added to a final concentration of 0.5 µg/ml. The running buffer used was 1x TBE with ethidium bromide added to a final concentration of 0.5 µg/ml. The gel was loaded with 5 µl PCR product mixed with 2 µl of loading dye, 5 µl PCR marker (Promega) was mixed with 2 µl loading dye and loaded onto the first and last lanes of the gel. The gels were run at 80 volts for 1 hour and visualised under UV.

5.3.1.6. Sensitivity with purified RNA

IPNV was propagated and purified, using methods described in Chapter 2. RNA was extracted from purified virus and RNase free TNE buffer as above. RNA concentration was measured using a Jenway 6405UV/Vis spectrophotometer, 10 µl of RNA was diluted 1:10 with RNase free water and the absorbance read at 260 and 280 nm. RNA concentration was calculated using the following formula from Ito and Joklik (1972):

O.D. at 260 nm x 60 x dilution factor (1 O.D. unit = 60 µg/ml double stranded RNA).

Viral RNA was then serially diluted to 15 ng/µl, 0.15 ng/µl, 1.5 pg/µl and 15 fg/µl in RNase free water, RNA extracted from TNE buffer acted as a negative control. RT-PCR was carried out. Once an indication of sensitivity was established RNA was further diluted from 15 ng/µl to 11.25, 7.5, 3.75, 1.5 and 0.75 ng/µl and the RT-PCR repeated. All products were visualised on an agarose gel as above.

5.3.1.7. Sensitivity using organ samples

Atlantic salmon kidneys were stored frozen at -70°C. IPNV was diluted 1:10 in HBSS (Gibco) from neat to 10^{-15}, 100 mg of kidney was spiked with 100 µl of alternate dilutions beginning with 10^{-1} to 10^{-15}, one kidney was spiked with 100 µl of HBSS to act as a negative control. To ascertain the exact virus concentration IPNV was titrated (Chapter 2) on the same day as the kidneys were spiked and RNA extracted. The kidneys used had been previously homogenised and placed onto CHSE-214 cells and had been found to be IPNV negative. RNA was then extracted using the Tri Reagent method and RT-PCR carried out.

5.3.1.8. Specificity

To ensure the primers were specific to IPNV various viruses were propagated and purified. The viruses used were a selection of those that were legally allowed to be grown in the virology laboratory at the Institute of Aquaculture. They were Infectious Salmon Anaemia Virus (ISAV), two strains of Piscine Nervous Necrosis Virus (PNNV(Nodavirus)), a Mediterranean strain from Seabass (*Dicentrarchus labrax*) and a Japanese strain from Striped Jack (*Pseudocaranx dentex*) and Blotched Snakehead Virus (BSNV).

ISAV was propagated on Salmon Head Kidney cells (SHK's) at 15°C. Cells were maintained in L15 Leibovitz without amino acids, with Glutamax (Gibco). Media was supplemented with 5% FBS (Gibco), 2mM L-Glutamine (Gibco), 40mM 2-Mercaptoethanol (BDH) and with 50 IU/ml penicillin, 50 µg/ml streptomycin and 100 µg/ml kanamycin (Gibco). SHK cells were infected with ISAV by pre-formed inoculation, media was removed from a 75cm^2 flask (Iwaki) of fully confluent SHK cells and retained, 3 ml ISAV was placed onto the cells and adsorbed at 15°C for 1 hour. After this time the conditioned media was replaced and the cells incubated at 15°C until full CPE was visible.

Both strains of Nodavirus and BSNV were propagated on Striped Snakehead fry/peduncle cells (SSN-1's) at 25°C. Cells were maintained in L15 Leibovitz without amino acids (Gibco), media was supplemented with 2 mM L-Glutamine (Gibco), 1x non-essential amino acids (Sigma) and 10% FBS for cell culture. FBS was lowered to 5% for virus propagation. SSN-1 cells were infected with both strains of Nodavirus by pre-formed inoculation, media was removed from a 75cm^2 flask (Iwaki) of fully confluent SSN-1 cells, 3 ml each Nodavirus was placed onto the cells and adsorbed for 30 minutes at 15°C. After this time L15 containing 5% FBS was added to the flask. SSN-1 cells were infected with BSNV by simultaneous inoculation of cells and virus. A 25cm^2 flask (Iwaki) of fully confluent SSN-1 cells was sub-cultured by washing twice with PBS Dulbeccos (without calcium, magnesium and sodium bicarbonate, Gibco) and cells harvested using trypsin-EDTA (1×, in HBSS without calcium and magnesium, Gibco), and split to a ratio of 1:3. Virus was inoculated at 1/10th of the volume of SSN-1 cell suspension (6 ml) in the flask and the cells/virus incubated at 25°C until full CPE was visible.

All viruses were harvested and clarified by centrifugation in a Denley BR401 refrigerated centrifuge at 1000 g for 10 minutes. Viruses were purified as in Chapter 2, Section 2.5. RNA was extracted (Section 5.2.1.3.) from the viruses and TNE buffer as a control using the above method. RNA from each virus and the TNE buffer was measured as above and the RNA serially diluted until each sample of viral RNA was at a concentration of 5 ng/µl. ISAV, both strains of Nodavirus and BSNV are single stranded RNA viruses, the concentration of ssRNA was calculated using the following formula:

O.D. at 260 nm x 40 x dilution factor (1 O.D. unit = 40 µg/ml ssRNA).

RT-PCR was then carried out and the products visualised on an agarose gel as above.

5.3.1.9. Detection of IPNV serotypes

To test whether the primers amplified every serotype of IPNV, each serotype was propagated by simultaneous inoculation of cells and virus, for method see Chapter 2. IPNV serotypes A1, A2 and A3 were propagated in CHSE-214 cells (Chapter 2); B1 was propagated in bluegill fibroblast cells (BF-2) at 15°C. BF-2 cells were cultured in EMEM without amino acids (Gibco) supplemented with 2 mM L-Glutamine (Gibco), 1x non-essential amino acids (Sigma) and 10% FBS (Gibco). All other serotypes, A4, A5, A6, A7, A8 and A9 were kindly donated by Keith Way at CEFAS and propagated on BF-2 cells. Viral RNA was extracted from cell culture supernatant (Section 5.2.1.3.) and RT-PCR carried out, products were visualised on an agarose gel as above. Amounts of template RNA were varied for each serotype from 0.5 µl to 3 µl.

5.3.2. IPNV polyclonal antiserum production

5.3.2.1. IPNV propagation

IPNV (Shetland isolate Br.) was propagated in CHSE-214 cells, 3 fully confluent 175 cm^3 flasks (Iwaki) were infected with IPNV by pre-formed inoculation for 30 minutes at 15°C (Chapter 2). Once complete CPE was visible the flasks were harvested and pooled, virus purified as in Chapter 2, however virus was re-suspended in PBS Dulbeccos (Gibco).

5.3.2.2. Antiserum production

Antiserum was produced in a New Zealand white rabbit, blood was collected prior to injection from the marginal ear vein. Blood was left to clot overnight at 4°C and centrifuged at 1250 g for 10 minutes in a Denley BR401 refrigerated centrifuge. Serum supernatant was removed and heat inactivated in a water bath at 56°C for 30 minutes and frozen in aliquots at -20°C. Purified virus was emulsified using equal volumes (500 µl) with Titermax adjuvant (Stratech Scientific Ltd.) and inoculated subcutaneously, 100 µl of the mixture was injected at 4 sites. A test bleed was taken from the marginal ear vein 14 days after the injection; blood was processed as above. A subcutaneous booster injection of purified virus and Titermax emulsion (100 µl at 4 sites) was given 4 weeks later. A further test bleed was taken from the marginal ear vein 7 days later and processed as above. The blood taken prior to injection was checked for IPNV antibodies by alpha neutralisation. Blood taken after the booster injection was also tested by alpha neutralisation to ensure the rabbit was responding to the injections.

The next booster injection was 4 months later; 400 µl of purified virus was intravenously injected into the ear vein, 7 days later the rabbit was anaesthetised and

blood taken by cardiac puncture. Blood was left to clot at room temperature for 1 hour and then at 4°C overnight, serum supernatant was then removed, the majority of the supernatant was heat inactivated as above for use in neutralisations. The rest was left untreated for ELISA; all supernatant was stored frozen at -20°C in aliquots.

5.3.2.3. Antiserum titre in cell culture

The titre of the antiserum was determined by alpha neutralisation. The alpha neutralisations were carried out in 96 well plates (Iwaki). For the pre-injection bleed and post booster bleed an indication of titre was all that was required, antiserum was diluted to a working concentration of 1:10 with HBSS plus 2% FBS, from which further dilutions of 1:20, 1:50, $1:10^{-2}$, $1:10^{-3}$, $1:10^{-4}$ and $1:10^{-5}$ were made. All wells except those in the first column were filled with 90 µl of HBSS plus 2 % FBS. Column one row one was filled with 100 µl of HBSS to act as a mock, all other wells in column one were filled with 100µl of IPNV. The virus and mock were then titrated 1:10 across the plate. Into the top two rows 90 µl of HBSS plus 2 % FBS was added, into the remaining 6 rows 90 µl of diluted antisera was added, one row per dilution. The plate was incubated at 15°C for 30 minutes, after this time 100 µl of CHSE-214 cells were added to each well. The plate was incubated at 15°C for 7 days after which time viral CPE was recorded.

To test the titre of the final bleed, an initial neutralisation as described above was carried out to gain an idea of the titre; two further neutralisations were then carried out to determine the exact titre. The antiserum was serially diluted with HBSS plus 2% FBS to 10^{-3}, 10^{-4} and 10^{-5}. The neutralisation was performed as above, except each dilution of antiserum was in duplicate, plus mock and virus only rows. Once a closer

estimation of titre was known the antiserum was further diluted using log 5 dilutions to $10^{-4.2}$ and $10^{-4.9}$, and to $10^{-4.7}$ using log 6 dilutions. The neutralisation was performed as above with each dilution of antiserum in duplicate, plus mock and virus only rows.

5.2.3.4. Antiserum specificity

The Shetland Br. isolate the antiserum was raised against was confirmed by neutralisation as serotype A2 (Sp.). The specificity of the antiserum was tested against homologous virus and IPNV reference strains A2 and A3 (Ab.) in duplicate using neutralisation titration. The neutralisation was performed as in Chapter 2 with one difference, the antiserum was diluted to a working concentration of 10^{-4}.

5.2.3.5. Polyclonal antibody purification

5.2.3.5.1. Sodium sulphate precipitation

Untreated polyclonal antiserum was brought to room temperature by heating for 5 minutes in a water bath at 25°C. Into 8 ml antiserum 14 % w/v sodium sulphate (Na_2SO_4, Sigma) was added and left in a water bath at 25°C for 5 minutes and then centrifuged for 15 minutes at full speed (21,000 g) in a IEC Micromax centrifuge. Supernatant was removed and the precipitate washed twice with a previously prepared solution of 14 % Na_2SO_4 in distilled water. Precipitate was dissolved in 2 ml PBS Dulbeccos (Gibco) at room temperature. Optical density of a 1:10 dilution of the suspension in PBS was read at 280 nm to determine protein concentration. An O.D. of 1.4 = 1 mg/ml protein.

5.2.3.5.2. Acid treatment of precipitated antiserum

PBS Dulbeccos (Gibco) was adjusted to pH 2.1 using hydrochloric acid (BDH). The appropriate volume of acidic PBS was added to the precipitated antiserum to give a final protein concentration of 3.6 mg/ml. The solution was left on a slow stirrer for 30 minutes, after this time the pH was brought up to 7.0 by addition of solid tris (Sigma). The purified acid treated antibody was stored in aliquots at -20°C.

5.3.3. ELISA

5.3.3.1. Optimisation

The ELISA plate (Immulon) was initially coated with the purified acid treated polyclonal antibody at concentrations of both 5 and 10 μg/ml and the AS-1 monoclonal (DiagXotics) the detector antibody at concentrations varying from 1:100-1:1000. This method was unsuccessful even when amplified with StreptAvidin horseradish peroxidase (HRP) (Scottish Antibody Production Unit (SAPU)). To ensure both antibodies were binding to the virus an indirect ELISA was carried out. ELISA plates were coated with 100 μl/well of 1 % poly-L-lysine (Sigma) diluted in coating buffer for an hour at room temperature. The plates were then washed 3 times with low salt wash buffer and 100 μl of IPNV placed into the wells and incubated overnight at 4°C. Purified polyclonal antibody was diluted to 1:500 and monoclonal antibody was diluted to 1:500, 100 μl of each was added to the plates.

The AS-1 monoclonal was then selected as the capture antibody and the polyclonal antibody as the detector antibody. The AS-1 monoclonal was diluted to 1:500; various dilutions of purified polyclonal antibody were tested including 5, 1, 0.1, and 0.01 μg/ml. Various blocking agents were then tested in an attempt to prevent non-specific binding,

1 % bovine serum albumin (BSA, Sigma), 3 % caesin (skimmed milk, Cadbury's Marvel) and 5 % gelatin (BDH) to block the monoclonal and 2.5 % gelatin as a diluent for the antigen, antibody and conjugate.

5.3.3.2. Sandwich ELISA

Prior to the ELISA kidney samples were heated to 40°C for 30 minutes to reduce levels of non-specific binding (Keith Way, pers. comm). A 96 well ELISA plate was coated with 100 µl of a 1:500 dilution of concentrated monoclonal antibody, AS-1 at 1.9mg/ml (DiagXotics) in coating buffer (pH 9.6, Appendix 1). The plate was incubated overnight at 4°C. After this time the plate was washed in low salt wash buffer (pH 7.3, Appendix 1) three times. The plate was then blocked with 250 µl of 3 % w/v caesin in distilled water for 1 hour at 37°C and the plate washed as above. The antigen was diluted in 3 % casein in low salt wash buffer and 100 µl added to each well and incubated at room temperature for an hour on a Titertek plate shaker. The plate was washed five times in high salt wash buffer (pH 7.6, Appendix 1) and incubated for 5 minutes on the last wash at room temperature. Purified polyclonal antibody was diluted in 3 % casein to a concentration of 0.5 µg/ml, 100 µl was added to each well and the plate incubated for an hour at room temperature. The plate was then washed as above. Biotin labelled donkey anti-rabbit IgG (SAPU) was diluted 1:1000 in conjugate buffer (Appendix 1) and 100 µl added to each well. The plate was incubated at room temperature for an hour and the above washing step was repeated. StreptAvidin HRP was diluted 1:1000 in conjugate buffer and 100 µl added to each well and the plate incubated at room temperature for 30 minutes. The plate was washed again as above. Substrate indicator solution was prepared by adding 3'3'5'5'-tetramethylbenidine dihydrochloride (TMB, Sigma) to 1:2 acetic acid:distilled water, 150 µl of this

chromogen solution was then added to 15 ml substrate buffer along with 5 µl of H_2O_2 (Sigma), 100 µl of this was added to every well and incubated at room temperature for 10 minutes. The reaction was stopped by addition of 50 µl of 2M H_2SO_4 (Sigma). The plate was read at 450 nm in a Titertek Multiskan Plus ELISA reader.

The sensitivity of the ELISA was evaluated using cell culture IPNV and a negative kidney homogenate spiked with a known amount of virus; negative controls of cell culture supernatant and negative kidney homogenate were used. The samples were assayed neat, diluted 1:10, 1:100 and 1:1000 and then transferred to the ELISA plate. At the same time the dilutions were titrated onto CHSE-214 cells to ascertain the exact titre of the virus (Chapter 2).

5.3.4. Immunohistochemistry

5.3.4.1. Optimisation

Initially the polyclonal rabbit antiserum produced in this study was used to stain paraffin embedded sections. Various dilutions of antiserum were tried, from 1:50 – 1:1000, no IPNV staining was visible. To attempt to unmask the antigens the slides were placed in the microwave at each incubation for 10 minutes, this too was unsuccessful using the polyclonal antiserum. The AS-1 monoclonal used for the ELISA was then tested at concentrations between 1:25 and 1:1000 without microwaving. A dilution of 1:500 was found to be optimal.

5.3.4.2. Immunohistochemistry

Paraffin embedded sections were used, tissues were fixed, processed and sectioned as in Chapter 2. Sections were dewaxed in xylene for 5 minutes and re-hydrated in 100 %

ethanol for 2 minutes and 70 % ethanol for 1.5 minutes and rinsed in distilled water. Rings were drawn around the sections with a PAP pen (BDH). All incubations were carried out in a humid chamber at room temperature.

Slides were incubated with 10 % v/v hydrogen peroxide in methanol for 10 minutes to block exogenous peroxidase activity. The slides were then washed 3x with Tris Buffered Saline (TBS, Appendix 1). Non-specific binding was blocked using normal goat serum (SAPU) diluted 1:10 in TBS for 10 minutes. The goat serum was tapped off and the slides washed as above. Monoclonal antibody AS-1 (DiagXotics) was diluted 1:500 from an original concentration of 1.9 mg/ml, placed onto sections and incubated for 60 minutes. Controls of negative tissue incubated with AS-1 and positive tissue incubated with PBS (Gibco) were also used. After this time the slides were washed as above. Biotin anti-mouse IgG (SAPU) was diluted 1:1000 in TBS, the sections coated and incubated for 60 minutes, sections were then washed as above. StreptAvidin HRP (SAPU) was diluted 1:1000 in TBS and placed onto the slides for 60 minutes, the sections were again washed as above. To visualise a reaction the slides were incubated for 15 minutes with 3'3 diaminobenzidine tetrachloride (DAB). A solution of 1 % H_2O_2 was prepared, 100 µl of this was added to 0.5 ml DAB (1.5 mg/ml) and 5 ml TBS. After 15 minutes the reaction was stopped by immersion of the slides in tap water.

Sections were counterstained with haematoxylin for 4 minutes, washed in tap water and immersed in Scotts tap water solution (STWS) for 30 seconds. Sections were then dehydrated in 70 % ethanol for 30 seconds, two baths of absolute alcohol for 2 minutes and 1.5 minutes. Finally slides were rinsed in xylene for 5 minutes and sections

coverslipped using pertex (Cellpath). Sections were examined under a light microscope, IPNV positive tissue stained brown.

5.4. Results

5.4.1. RT-PCR

5.4.1.1. Sensitivity

Using purified IPNV RNA the detection limit of the RT-PCR using primer set PrD was found to be 1.5 ng/µl (Figure 5.1).

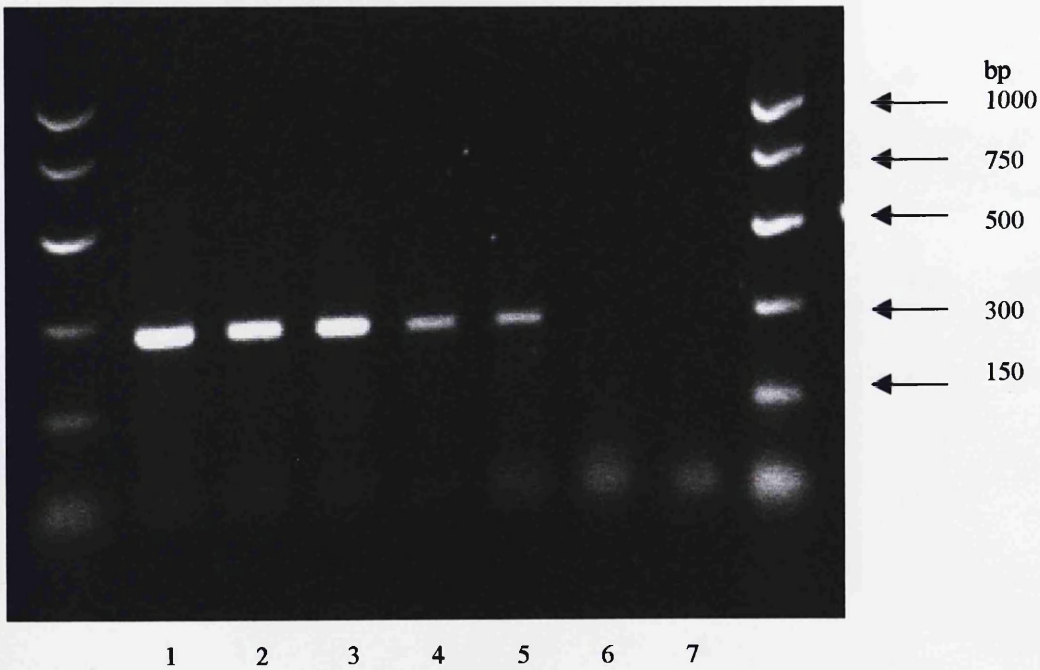


Figure 5.1: Sensitivity of RT-PCR using primer set PrD. Lane 1: 15ng/µl; lane 2: 11.25 ng/µl; lane 3: 7.5 ng/µl; lane 4: 3.75 ng/µl; lane 5: 1.5 ng/µl; lane 6: 0.75 ng/µl; lane 7: TNE buffer extract.

Using purified IPNV RNA the detection limit of the RT-PCR using primer set PrF was found to be 0.75 ng/µl (Figure 5.2).

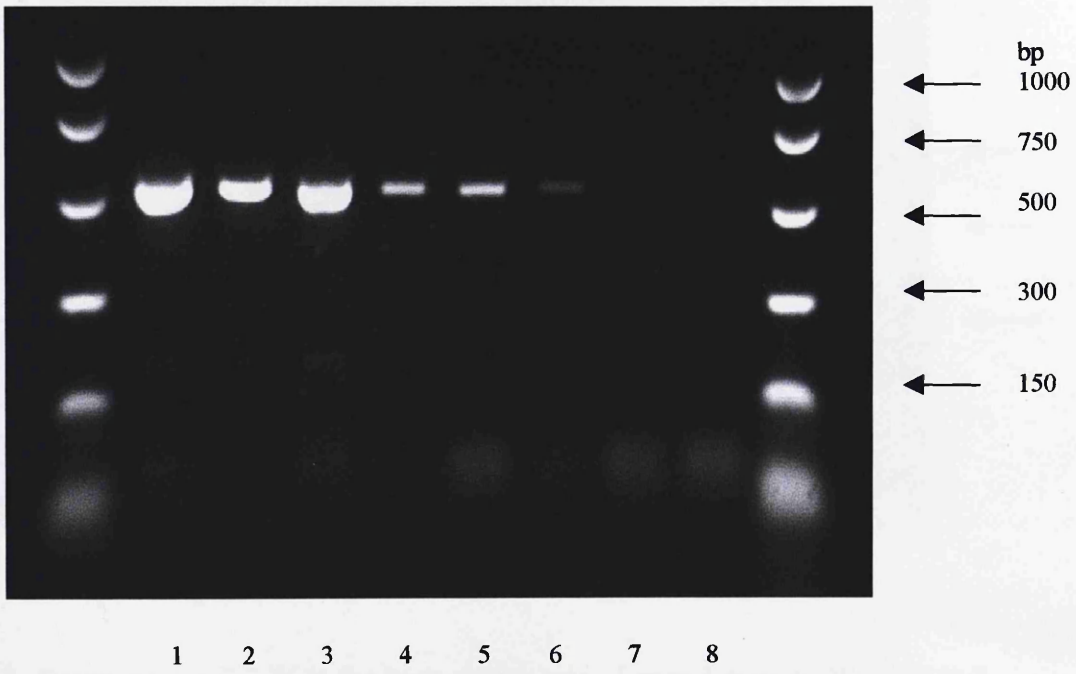


Figure 5.2: Sensitivity of RT-PCR using primer set PrF. Lane 1: 15ng/µl; lane 2: 11.25 ng/µl; lane 3: 7.5 ng/µl; lane 4: 3.75 ng/µl; lane 5: 1.5 ng/µl; lane 6: 0.75 ng/µl; lane 7: 0.1 ng/µl; lane 8: TNE buffer extract.

Primer set PrF was found to be slightly more sensitive than PrD.

5.4.1.2. Specificity

Both primer sets were found to only amplify IPNV RNA (Figure 5.3), no band was visible when using RNA from ISAV, two strains of Nodavirus and BSNV.

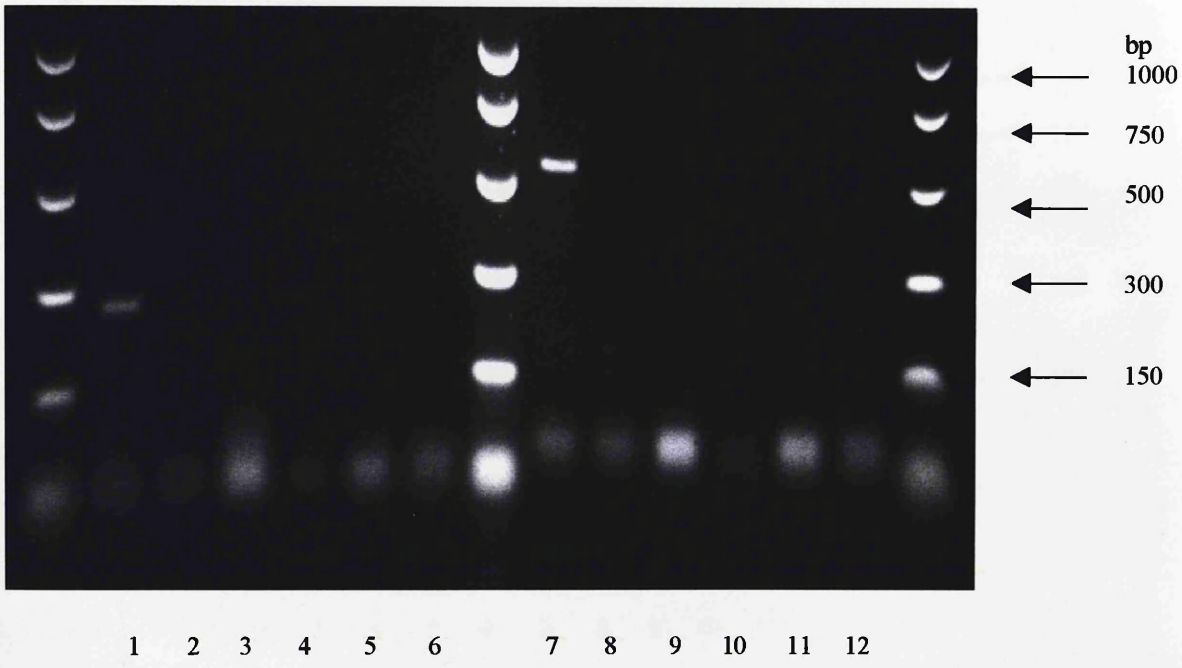


Figure 5.3: Specificity of RT-PCR for both primer sets. Lanes 1-6 using PrD, lanes 7-12 using PrF. Lanes 1 and 7: IPNV; lanes 2 and 8: ISAV; lanes 3 and 9: Maltese Nodavirus; lanes 4 and 10: Japanese Nodavirus; lanes 5 and 11: BSNV; Lanes 6 and 12: TNE buffer extract.

Primer set PrD was found to amplify 7/10 serotypes of IPNV (Figure 5.4), A1, A2, A4, A6, A7 and A9. The primer set also amplified A5 however the product at this serotype was very faint.

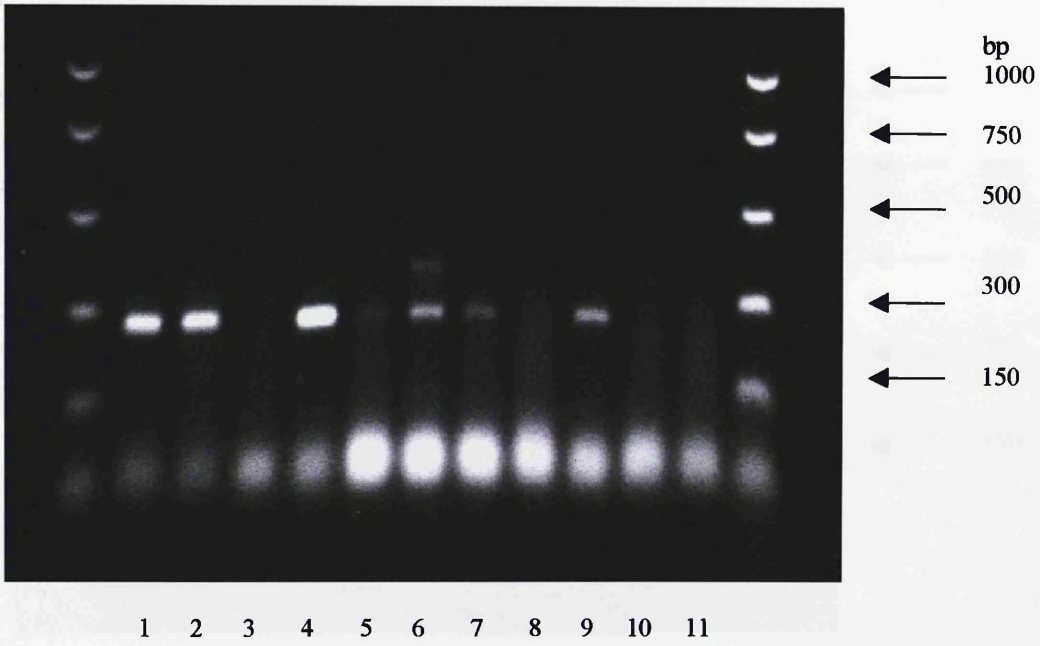


Figure 5.4: Amplification of IPNV serotypes with primer set PrD. Lane 1: A1; lane 2: A2; lane 3: A3; lane 4: A4; lane 5: A5; lane 6: A6; lane 7: A7; lane 8: A8; lane 9: A9; lane 10: B1; lane 11: BF-2 cell culture supernatant.

Primer set PrF was found to amplify 6/10 serotypes of IPNV (Figure 5.5), A1, A2, A3, A5, A7 and A8. Faint bands were also visible at A4 and A9 however amplification was not consistent with these serotypes.

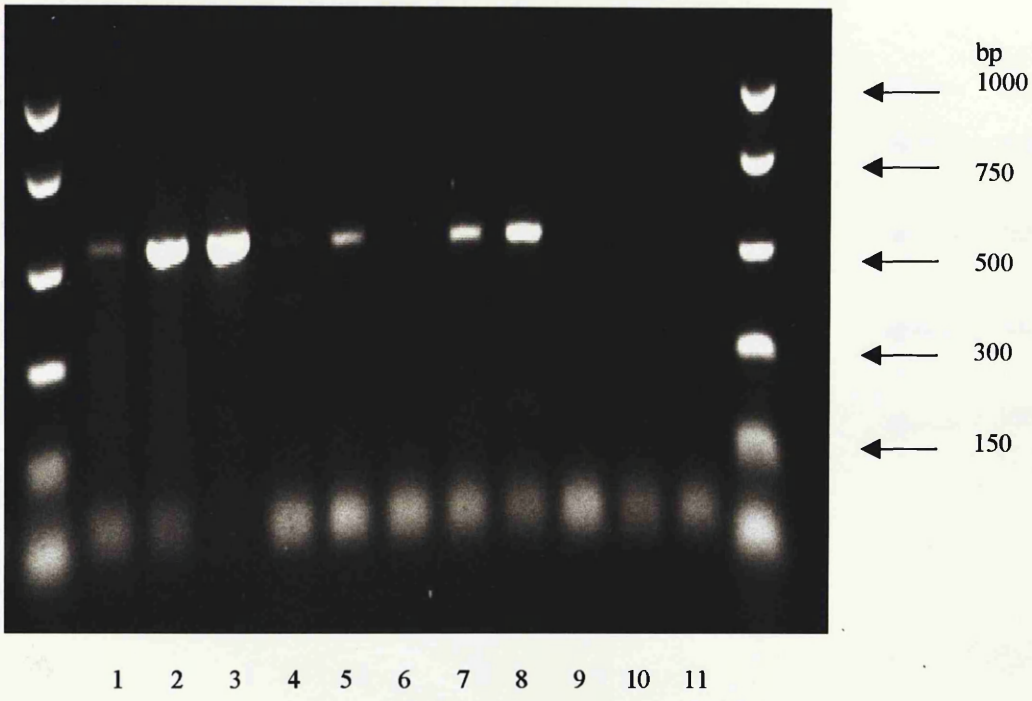


Figure 5.5: Amplification of IPNV serotypes with primer set PrF. Lane 1: A1; lane 2: A2; lane 3: A3; lane 4: A4; lane 5: A5; lane 6: A6; lane 7: A7; lane 8: A8; lane 9: A9; lane 10: B1; lane 11: BF-2 cell culture supernatant.

Neither primer set PrD nor PrF amplified serotype B1. However using both primer sets it was possible to amplify 9/10 serotypes of IPNV.

5.4.1.3. Adaptation of RT-PCR for use with tissue

Amplification of IPNV from kidney using the tissue extraction kit was repeatedly unsuccessful, even using the most sensitive primer set PrF (Figure 5.6). Note the amplification of spurious non-specific bands rather than IPNV in lane 2.

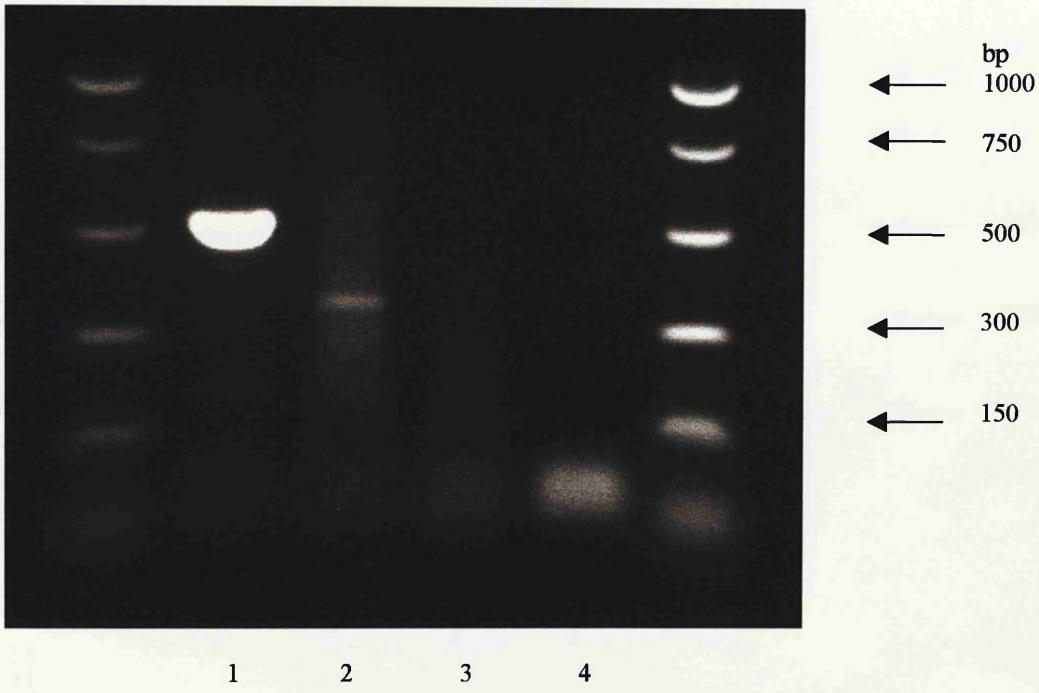


Figure 5.6: Amplification of RNA extracted using the tissue extraction kit from IPNV spiked kidney tissue using primer set PrF. Lane 1: A2 positive control; lane 2: 2 µl spiked kidney RNA; lane 3: 0.5 µl spiked kidney RNA; Lane 4: HBSS spiked kidney RNA.

Amplification of IPNV RNA from kidney was successful using Tri Reagent to extract IPNV RNA from kidney spiked with high titre virus (Figure 5.7).

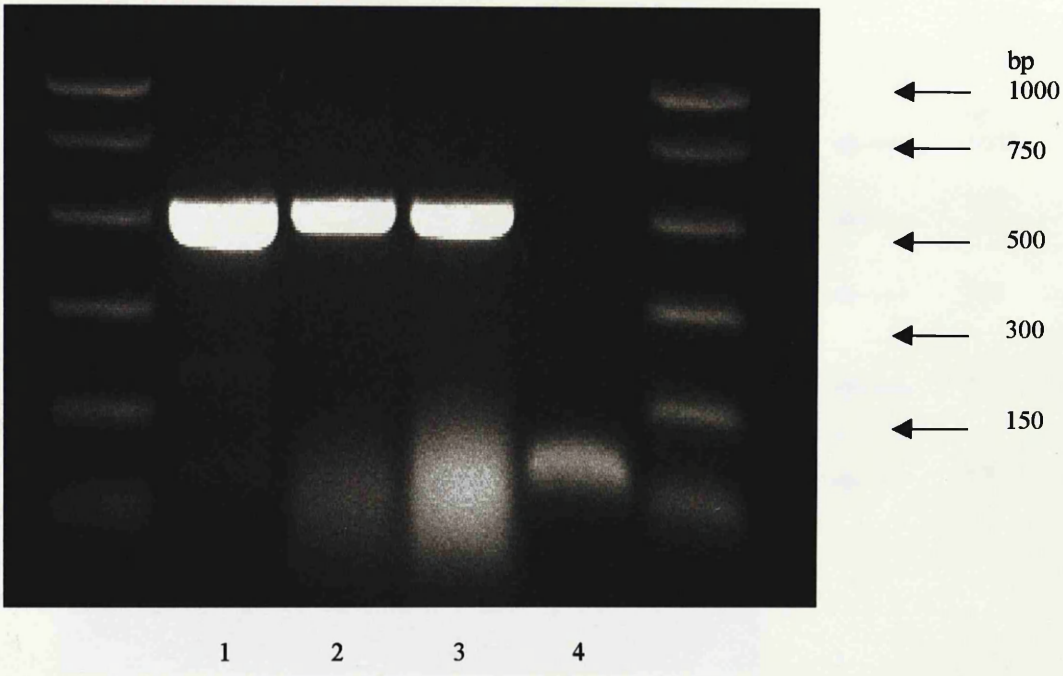


Figure 5.7: Amplification of RNA extracted using Tri Reagent from kidney tissue spiked with 10^{12} $TCID_{50}$ IPNV using primer set PrF. Lane 1: A2 positive control; lane 2: 1 µl spiked kidney RNA; lane 3: 2 µl spiked kidney RNA; lane 4: HBSS spiked kidney RNA.

5.4.1.3. Sensitivity using tissue

Amplification of IPNV from kidney spiked with various dilutions of virus was unsuccessful with primer set PrD even at dilutions as low as 10^{-1} (10^{8} $TCID_{50}$/ml) (Figure 5.8).

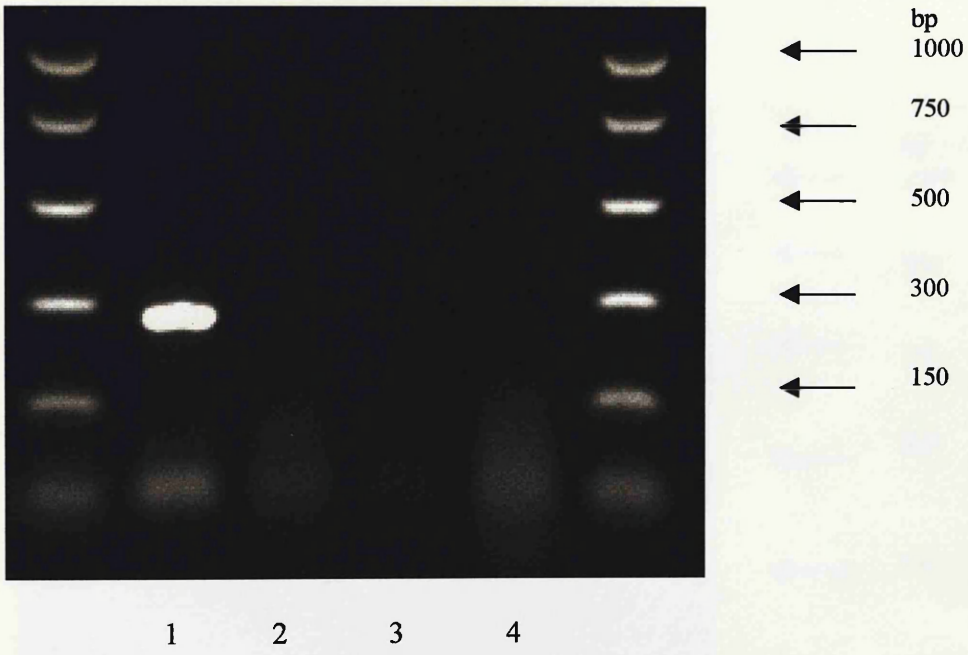


Figure 5.8: Sensitivity of primer set PrD using IPNV spiked kidney RNA. Lane 1: A2 positive control; lane 2: 10^{-1} IPNV; lane 3: 10^{-3} IPNV; lane 4: HBSS spiked kidney RNA.

However amplification was successful using primer set PrF. A product was visualised only at the lowest dilution of 10^{-1}, this equates to 10^8 $TCID_{50}$ IPNV/ml but not at 10^6 $TCID_{50}$/ml (Figure 5.9). Alternative dilutions of IPNV were amplified therefore the RT-PCR was sensitive to between 10^7 and 10^8 $TCID_{50}$/ml.

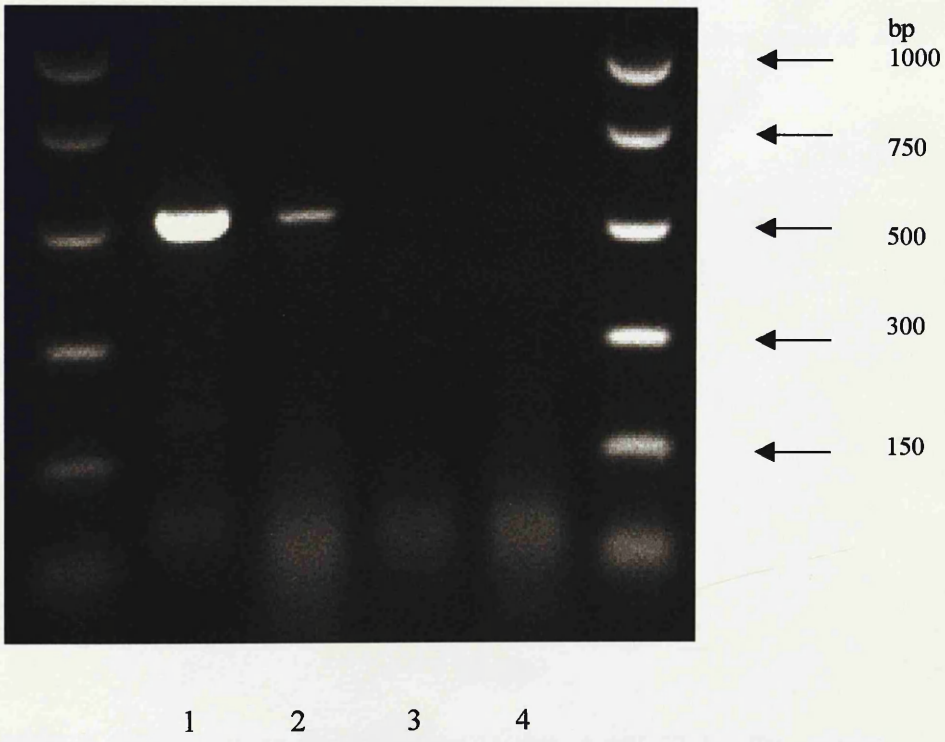


Figure 5.9: Sensitivity of primer set PrF using IPNV spiked kidney RNA. Lane 1: A2 positive control; lane 2: 10^7 IPNV; lane 3: 10^5 IPNV; lane 4: HBSS spiked kidney RNA.

5.4.2. Antiserum specificity and titre

The polyclonal antiserum was found not to neutralise IPNV serotype A3 (Ab.) reference strain but was found to neutralise IPNV A2 (Sp.) reference strain. The 50 % neutralisation titre of the antiserum using homologous virus was found to be 10,000.

5.4.3. ELISA

The sandwich ELISA was unsuccessful using the purified polyclonal antibody as the capture antibody with different dilutions of IPNV (Figure 5.10). There was very little difference in the optical density O.D. between IPNV and the negative control of EMEM.

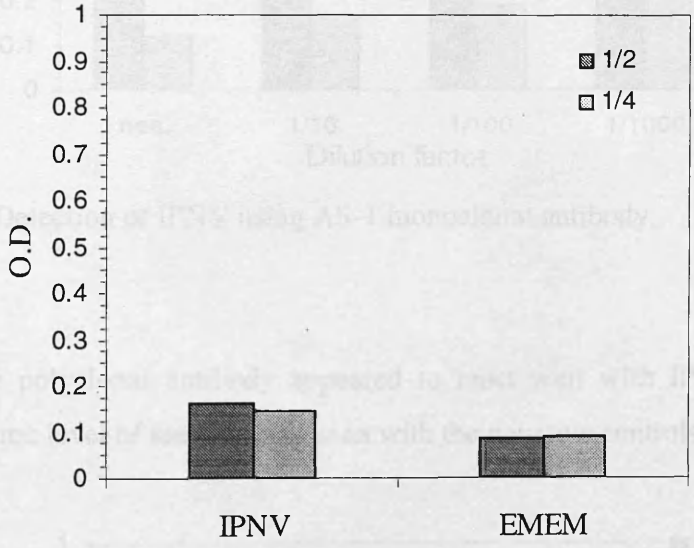


Figure 5.10: Sandwich ELISA using 5 µg/ml polyclonal antibody as the capture antibody at two dilutions of IPNV.

The AS-1 monoclonal antibody was shown to react well with IPNV in an indirect ELISA at a dilution of 1:500 (stock concentration of 1.9 mg/ml). However non-specific binding in the negative controls was above an O.D. of 0.1 and appeared to increase with higher dilutions of EMEM.

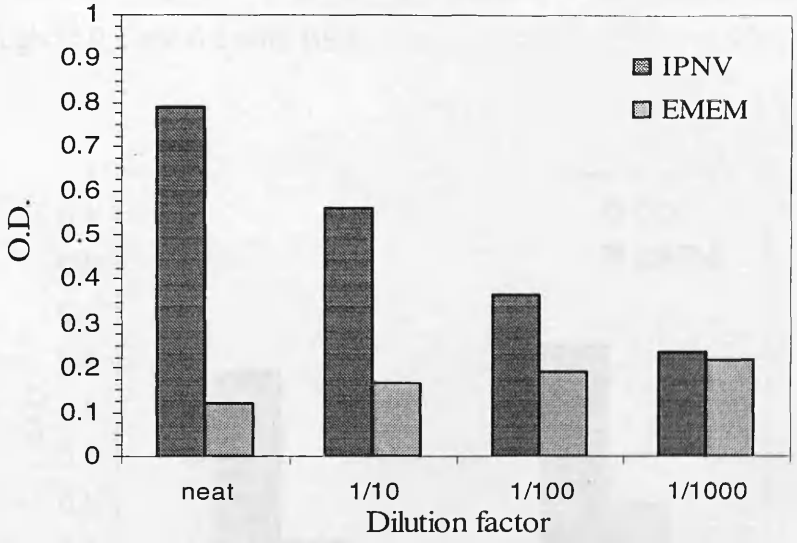


Figure 5.11: Detection of IPNV using AS-1 monoclonal antibody.

Although the polyclonal antibody appeared to react well with IPNV in an indirect ELISA the same level of reaction was seen with the negative controls of EMEM (Figure 5.12).

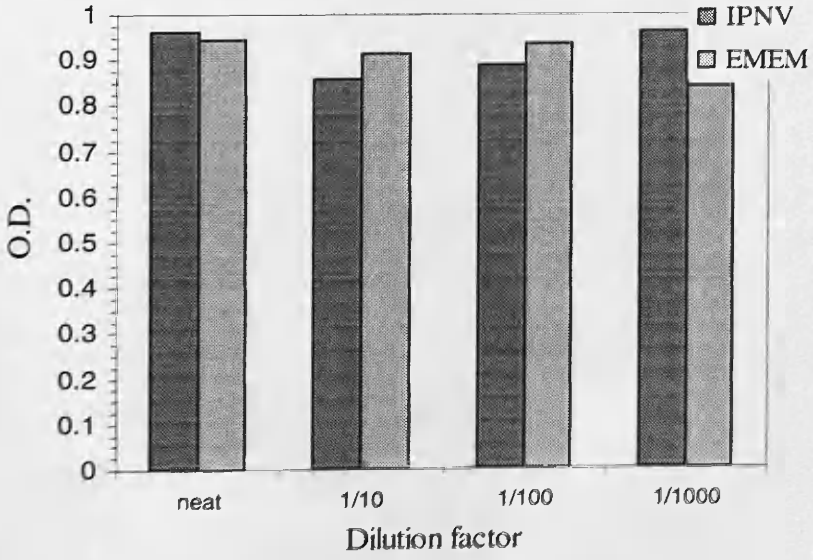


Figure 5.12: Detection of IPNV using 10 µg/ml polyclonal antibody.

Changing the capture antibody to the monoclonal antibody at a dilution of 1:500 produced a good reaction with IPNV (Figure 5.13). A comparison of 1 % BSA and 2.5 % gelatin as blocking agents showed that neither BSA nor gelatin were particularly efficient at blocking non-specific binding. Reducing the polyclonal antibody concentration to 1 μg/ml did little to lower non-specific binding, binding with gelatin was as high as 0.3 and 0.2 with BSA.

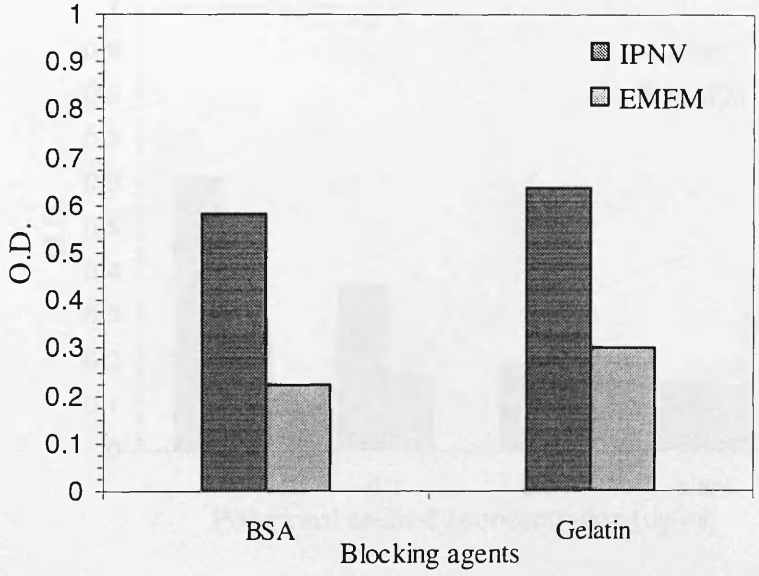


Figure 5.13: Sandwich ELISA using the AS-1 monoclonal as the capture antibody comparing 1 % BSA and gelatin as blocking agents.

To attempt to reduce non-specific binding the polyclonal antibody was titrated out from 1 µg/ml to 0.01 µg/ml using 1 % BSA as the blocking agent. Reducing the concentrations of polyclonal antibody appeared to have little effect on non-specific binding, the blanks containing no antibody were just as high as those with antibody and still above 0.1.

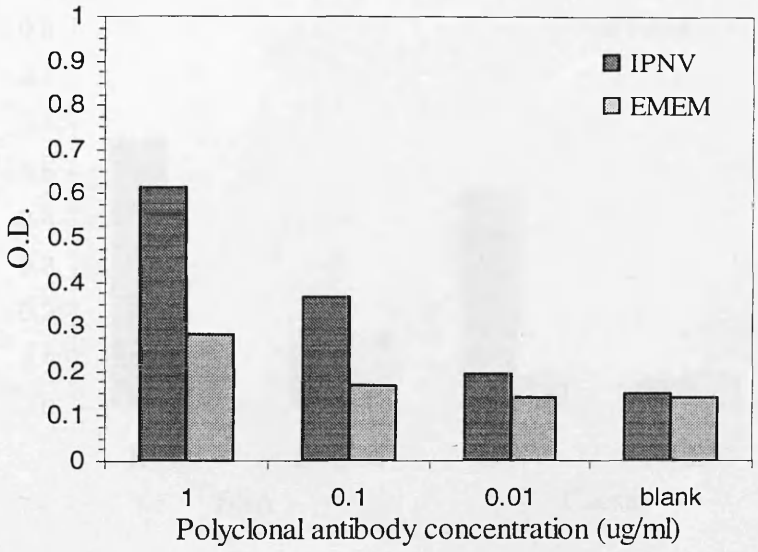


Figure 5.14: A comparison of different concentrations of polyclonal antibody.

The concentration of polyclonal antibody chosen based on the earlier titration was 0.5 µg/ml. Using caesin as a blocking agent at this concentration of polyclonal was successful at blocking non-specific binding (Figure 5.15), which was below an O.D. of 0.1 (0.05-0.07).

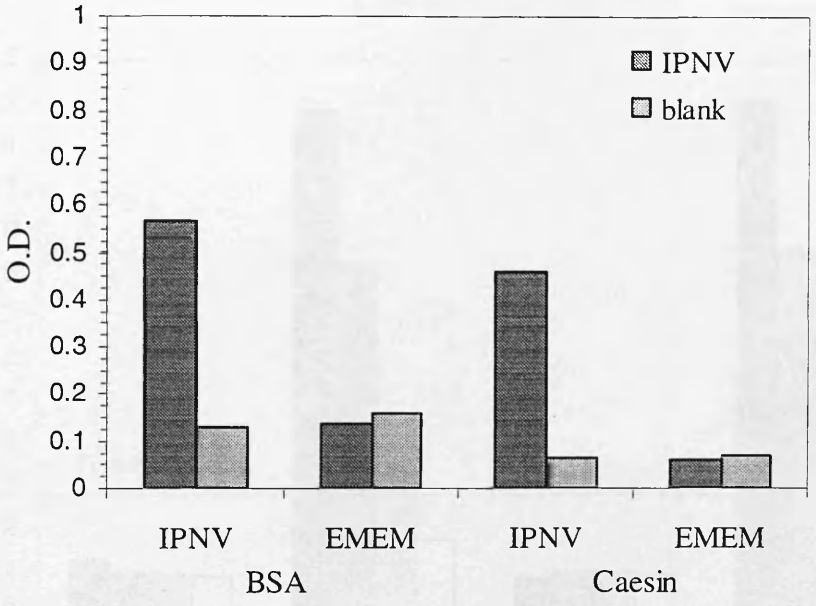


Figure 5.15: Comparison of 1 % BSA and 3 % caesin as blocking agents.

The sensitivity of the ELISA was little different using cell cultured IPNV or spiked kidney homogenate, both were sensitive to $10^{12.5}$ $TCID_{50}$/ml (the 1:100 dilution in Figure 5.16). The sensitivity threshold of the ELISA was 3 times the O.D. of the negative controls, in this case 0.285.

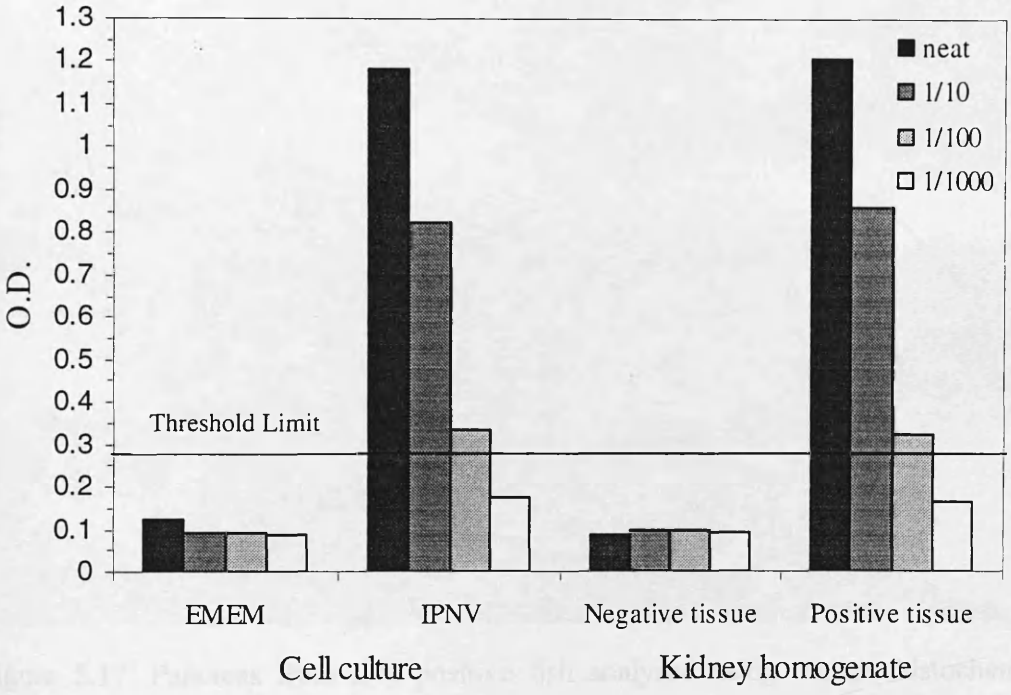


Figure 5.16: Sensitivity of the sandwich ELISA in cell culture and spiked kidney homogenate.

5.4.4. Immunohistochemistry

IPNV was successfully identified using AS-1 monoclonal antibody, lesions in the pancreas tissue stained positive for IPNV (Figures 5.17 and 5.20).

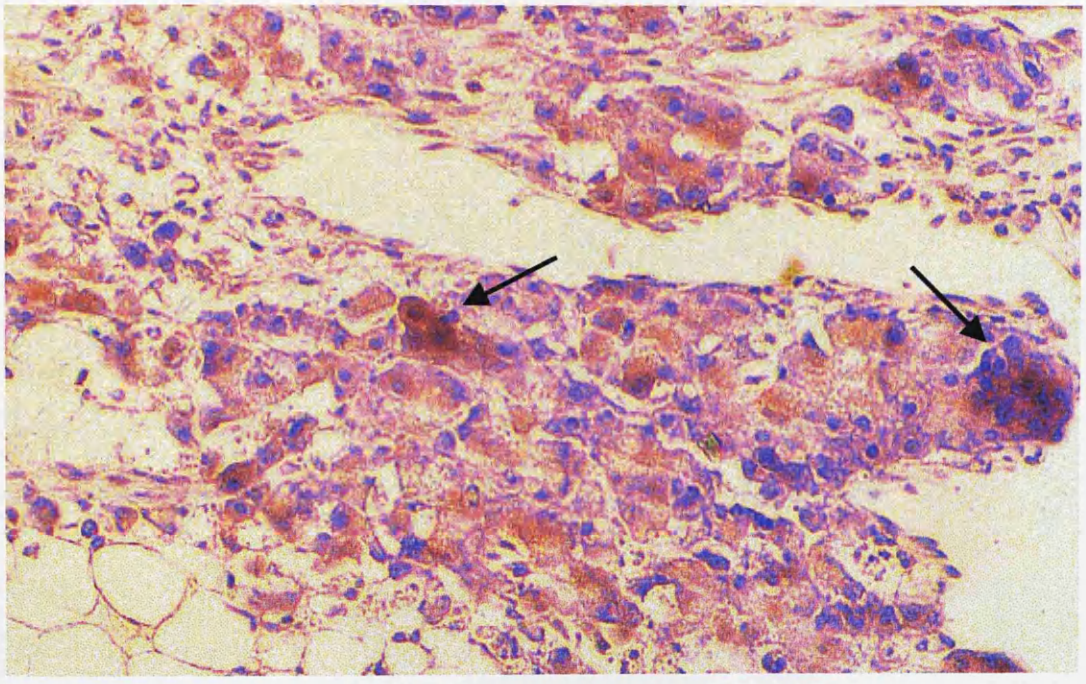


Figure 5.17: Pancreas from IPN positive fish analysed using immunohistochemistry with the AS-1 monoclonal antibody. Magnification: x20. Note areas of intense IPNV staining (arrows).

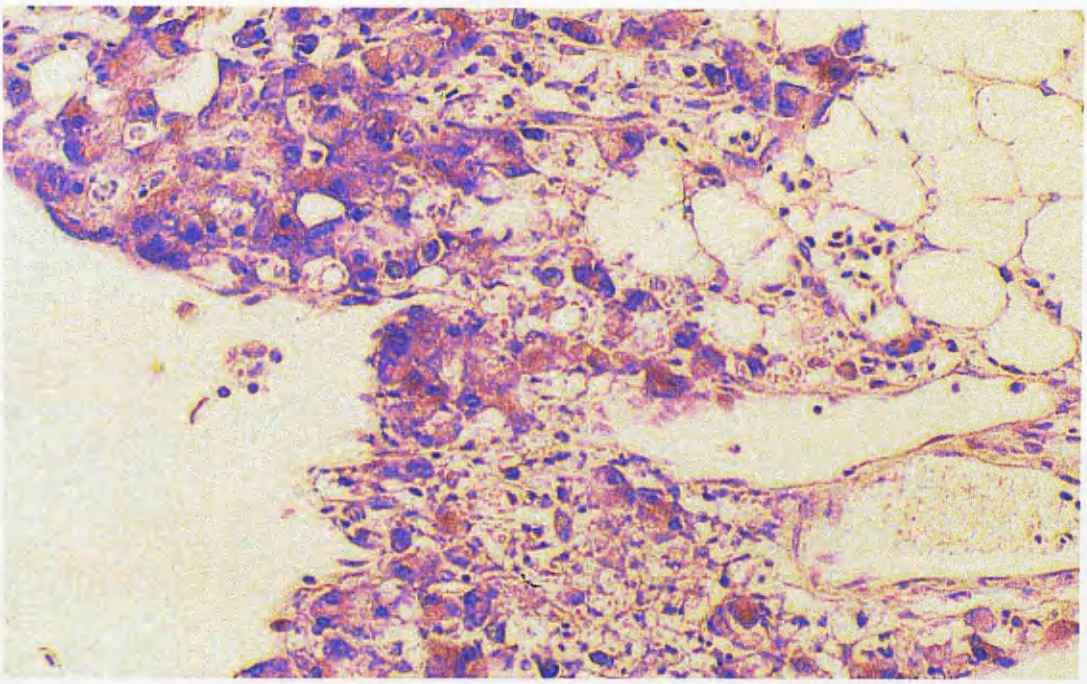


Figure 5.18: Pancreas from IPNV positive fish analysed by immunohistochemistry using PBS. Magnification: x20. Note the lack of IPNV specific staining.

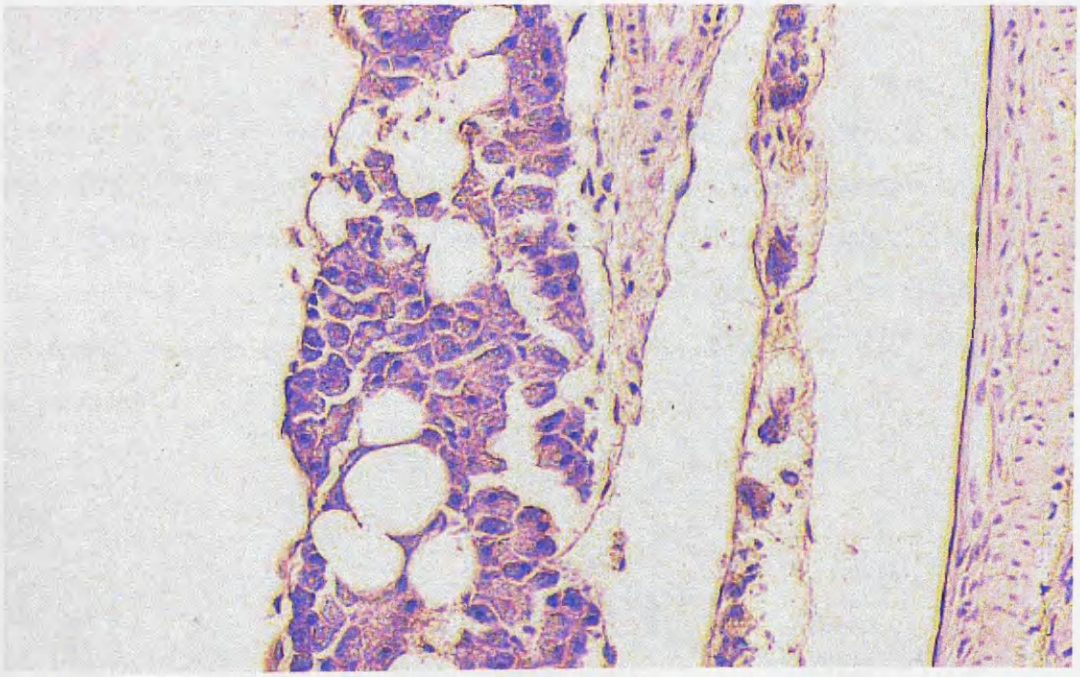


Figure 5.19: Pancreas from IPN negative fish analysed by immunohistochemistry with the AS-1 monoclonal antibody. Magnification: x20.

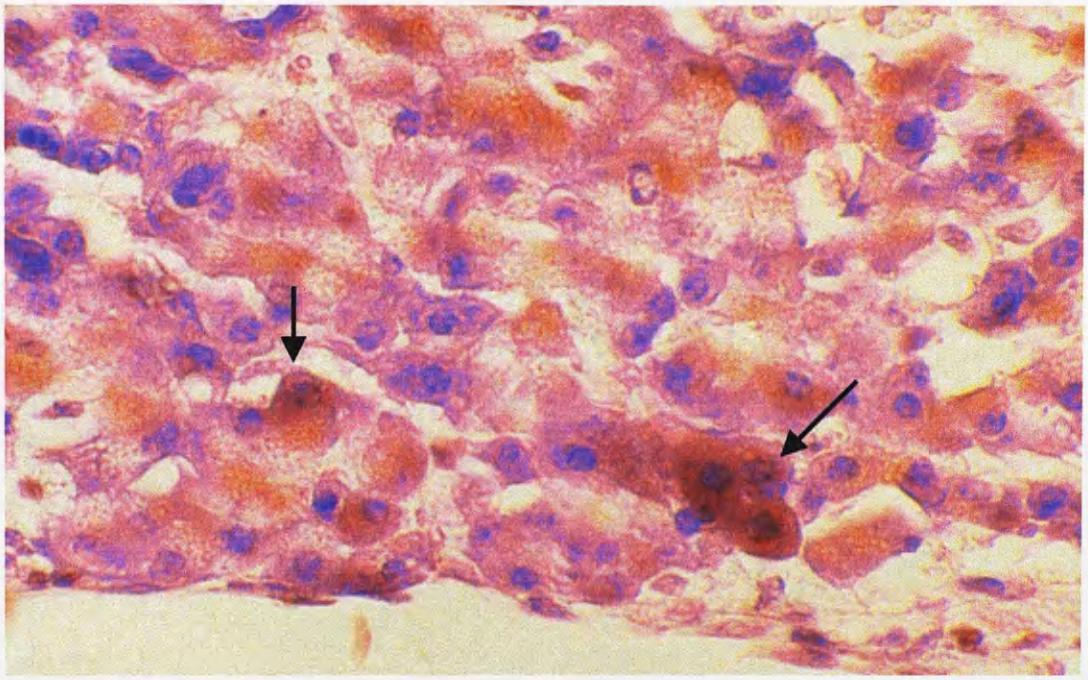


Figure 5.20: Pancreas from IPN positive fish analysed by immunohistochemistry with the AS-1 monoclonal antibody. Magnification: x40. Note intense staining of exocrine pancreas cells (arrows).

A certain amount of background staining was present in the IPNV positive tissue stained with PBS upon examination of the slide under high magnifications (Figure 5.21). There was also a certain amount of staining in the IPNV negative tissue stained with the AS-1 monoclonal (Figure 5.22). This was mainly staining of the fat cell membranes that was considered unlikely to be confused with specific IPNV staining in the pancreas.

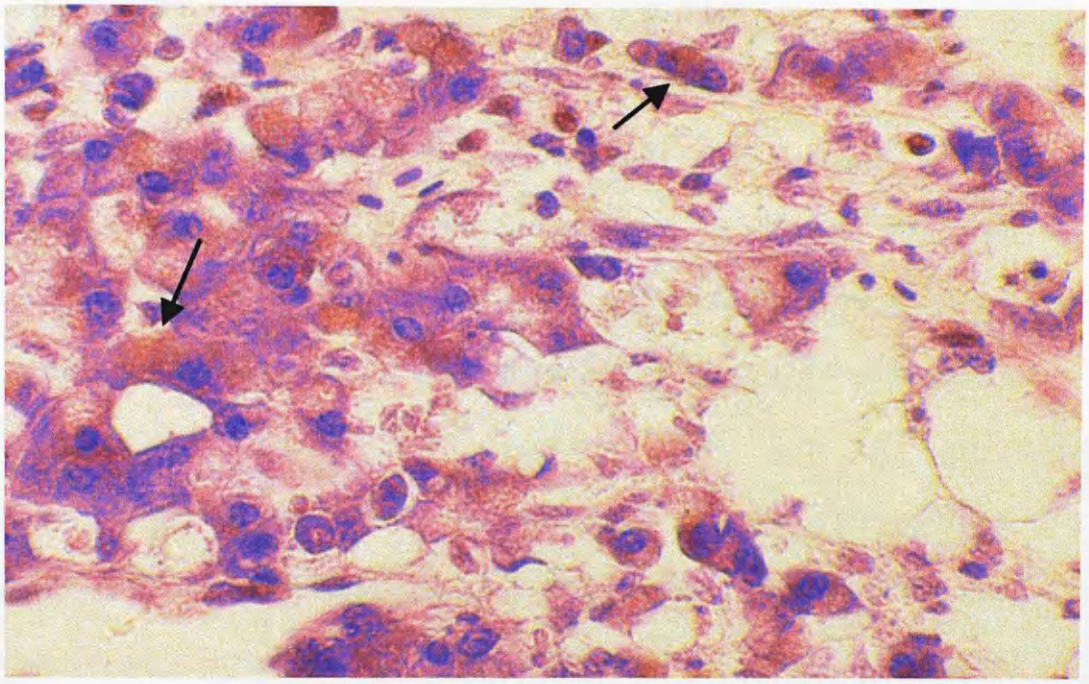


Figure 5.21: Pancreas from IPN positive fish analysed by immunohistochemistry with PBS. Magnification: x40. Note slight non-specific staining in exocrine pancreas cells (arrows).

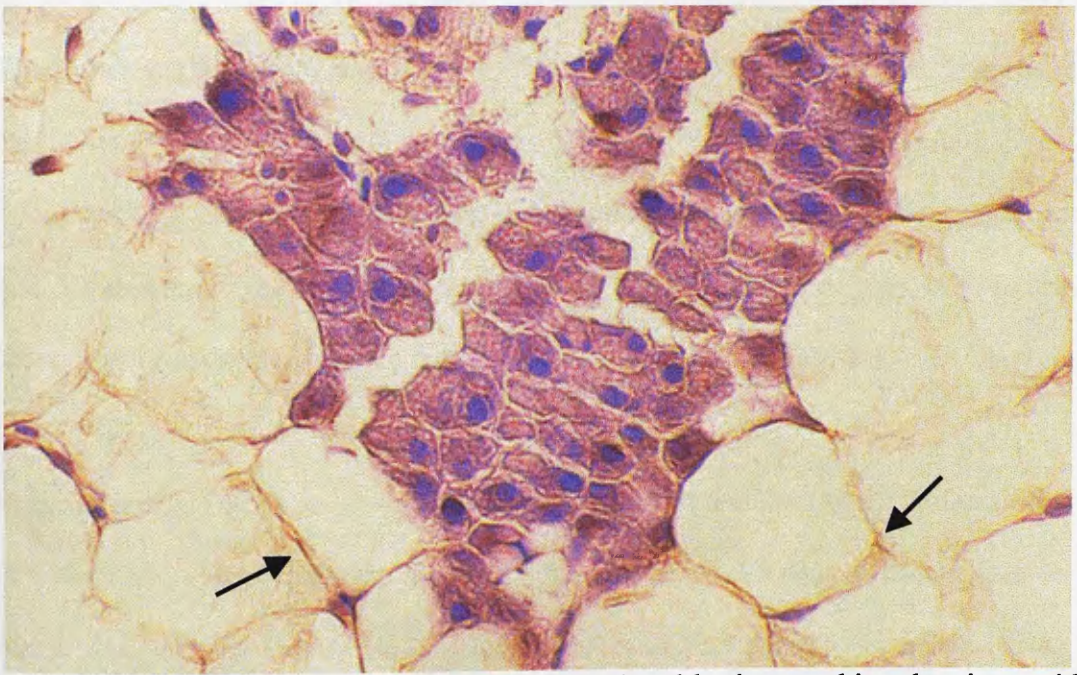


Figure 5.22: Pancreas from IPN negative fish analysed by immunohistochemistry with the AS-1 monoclonal antibody. Magnification: x40. Note non-specific staining of membranes in negative tissue (arrows).

5.5. Discussion

Cell culture was found to be the most sensitive detection method for IPNV in the present study. The process of simultaneous inoculation of IPNV and CHSE-214 cells is likely to be more sensitive than the inoculation of IPNV onto pre-formed cell monolayers. In a simultaneous inoculation the cells are slightly compromised giving the virus a greater opportunity for infection, particularly with a low level of infection.

The sensitivity of RT-PCR is often measured using pure viral RNA and to enable comparison the sensitivity using purified IPNV RNA was carried out in this study. In the present study primer sets PrD and PrF were found to be sensitive to 1.5 ng and 750 pg respectively, Wang *et al.* (1997) found primer set PrD to be sensitive to 15 fg and PrF to 15 pg. Lopez-Lastra *et al.* (1994) examined 5 primer sets and found the most sensitive primer set to amplify 1 pg of purified IPNV RNA. The adaptation of the RT-PCR to RT-PCR beads appears to have reduced the sensitivity using purified IPNV RNA, however whilst Wang *et al.* (1997) found PrD to be the most sensitive the present study found PrF to be the most sensitive. Wang *et al.* (1997) used the A1 (West Buxton) serotype to establish sensitivity; the present study used the A2 (Sp.) serotype. Slight differences in the nucleotide sequence of the two serotypes may explain the difference in amplification efficiency.

The use of purified RNA to assess sensitivity is not representative of the utility of the RT-PCR in the field where the assay is likely to be much less sensitive due to contaminants such as kidney RNA. Many publications establish sensitivity using purified RNA but do not do the same for tissue homogenates (Lopez-Lastra *et al.*, 1994, Wang *et al.*, 1997). RT-PCR may potentially be as sensitive as cell culture for detection

of IPNV, however of the 2 primer sets examined in this study PrD failed to amplify the virus at all from spiked kidney homogenate and primer set PrF was only sensitive to between 10^7 and 10^8 $TCID_{50}$/ml. Blake, Schill, McAllister, Lee, Singer and Nicholson (1995) assessed the sensitivity of the RT-PCR compared to cell culture for detection of IPNV, they concluded that with one primer set the RT-PCR was as accurate as cell culture isolation for detecting virus in kidneys from asymptomatic carrier fish. Blake *et al.* (1995) assessed 4 different primer sets and established sensitivity for both cell culture and tissue homogenates to be between $10^{3.2}$ and $10^{4.6}$ plaque forming units (PFU)/ml. Assessment of viral titre using PFU's is not directly comparable to the $TCID_{50}$ method and unfortunately the two sensitivities cannot be compared.

It is likely that the RNA tissue extraction kit initially used in this study was ineffective at removing the high levels of DNA, RNA and protein present in the kidney as demonstrated by the very viscous nature of the tissue homogenate. The Tri reagent RNA extraction method removed all protein and DNA, only total RNA was pelleted and re-suspended. The high amount of RNA present in the kidney is likely to be responsible for inhibition of amplification of the RT-PCR from tissue. An alternative organ could be sought for IPNV isolation for use in RT-PCR, unfortunately alternatives such as the pancreas and gut or spleen are also very rich in nucleic acids and may harbour too little virus, particularly for detecting carrier status.

The 10 minute denaturation step of dsRNA and primers prior to RT-PCR was adapted from Davis and Boyle (1990) and Lopez-Lastra *et al.* (1994). A factor that may affect the sensitivity of the RT-PCR is this denaturation step; the amount of dsRNA split into ssRNA and annealed to the primers may vary with each PCR due to the high secondary

structure of dsRNA. Lopez-Lastra *et al.* (1994) assessed a number of temperatures for the denaturation step and found that a proteinase K digestion of the dsRNA followed by 10 minutes denaturation at 104°C gave the best results. Wang *et al.* (1997) denatured IPNV dsRNA at 110°C for 10 minutes whilst Davis and Boyle (1990) denatured IBDV dsRNA at 100°C for 10 minutes. Dobos (1995) states that the IPNV dsRNA genome has a Tm (melting point) of 89°C, therefore heating to any temperature above 89°C should denature the dsRNA. It is possible that with increasing temperatures above 89°C the more dsRNA is denatured and the more sensitive the RT-PCR. In the present study denaturation was 100°C for 10 minutes, it is possible that the RT-PCR may be more sensitive with denaturation at a higher temperature.

Staining of agarose gels with ethidium bromide is not particularly sensitive and to increase the detection limit of IPNV cDNA may be visualised using polyacrylamide gel electrophoresis (PAGE) and stained with silver stain (Berry and Samuel, 1982, Herring, Inglis, Ojeh, Snodgrass and Menzies, 1982). Berry and Samuel (1982) assessed the detection limit of dsRNA using PAGE, staining with silver stain and ethidium bromide. They found that silver stain was between 10-30 times more sensitive than ethidium bromide. Lopez-Lastra *et al.* (1994) established the detection limit of the RT-PCR to be 1 pg using PAGE, this is more sensitive than the RT-PCR described in the present study however Wang *et al.* (1997) found the sensitivity with primer set PrD to be 15 fg visualising cDNA on agarose gels. Lopez-Lastra *et al.* (1994) increased the sensitivity of their RT-PCR with the use of a nested PCR, Suzuki, Hosono and Kasuda (1997) increased the sensitivity of their IPNV RT-PCR from 1 pg to 1 fg using nested PCR. However a nested PCR increases the chances of obtaining false positive results.

It is possible that decreasing the volume of RNase free water used to re-suspend the RNA from 100 µl to 50 µl may increase the sensitivity, yet this will also further concentrate total RNA and may only inhibit the assay further. Performing the RT-PCR in two steps, an RT step and a PCR step may also increase sensitivity although this too has drawbacks, the time taken for the assay is increased along with the potential for contamination. Alternatively, dsRNA has been found to be resistant to RNase A digestion in the presence of high salt levels unlike ssRNA and it is possible to remove the ssRNA component in this way (Nicholson, 1996). This is likely to be the most effective method for increasing the sensitivity of the RT-PCR and will not significantly increase the time taken for the RNA extraction. This method should be examined for future use.

Despite the low sensitivity of the RT-PCR it was found to be specific to IPNV; ISAV, BSNV and two strains of Nodavirus did not produce a product. These viruses were chosen for two reasons; they could be legally cultured at the Institute and they were viruses that may co-exist with IPNV around the world. Wang *et al.* (1997) also concluded that the primers were specific to IPNV since amplification of IHNV and IBDV failed to produce a product.

In light of the poor sensitivity of the RT-PCR it is unlikely the test could be used routinely as a diagnostic method, nevertheless it does have potential to confirm IPNV infection upon the appearance of CPE in cell culture. This does have its limitations since both sets of primers were shown not to amplify all serotypes of IPNV; this depends on the serotypes of the virus present in the area of isolation. However, the use of both primer sets does allow for amplification of all 9 serotypes of IPNV within

serogroup A. TV-1 is a shellfish isolate and was not amplified by either primer set, it is the only representative to date of serogroup B and is present in the UK. RT-PCR does not give any information on the serotype of IPNV and it is likely that a titration neutralisation will still be required. The PCR does allow for a rapid diagnosis of infection and results may be determined in one day rather than 7, the time required for a neutralisation titration.

The sandwich ELISA was found to be less sensitive than the RT-PCR, the detection limit was $10^{12.5}$ $TCID_{50}$/ml, although the sensitivity of the assay does not decrease when comparing cell cultured IPNV to spiked tissue homogenates. ELISA tests can never be sensitive enough to detect the very low virus levels typical of carrier fish, but are able to detect virus in infected cell cultures (Davis *et al.*, 1994). However the ELISA developed in this study appears not to detect even high titres of virus in cell culture. ELISA protocols have previously been published with sensitivities of 10^6 PFU/ml, 10^5 $TCID_{50}$/ml and 10^3 $TCID_{50}$/ml (Dixon and Hill, 1983a, Davis *et al.*, 1994, Vazquez Branas, Coll Morales and Estepa, 1994).

It is possible that one of the two antibodies used in the ELISA may be a low avidity antibody. The polyclonal antibody was found to be ineffective at staining IPNV in immunohistochemistry and this may indicate that it is the polyclonal with a low avidity and is removed with the rigorous washes performed in both the ELISA and immunohistochemistry. The epitopes the polyclonal antibody reacts with may be investigated using Western blotting. Alternatively since the epitopes the polyclonal antibody reacts with were not assessed in this study it is possible that the two antibodies are competing for the same sites. The AS-1 monoclonal reacts with VP2, this is

considered to be the major protein involved in the infectivity of IPNV (Caswell-Reno *et al.*, 1986). It is possible that since both antibodies are neutralising antibodies they may both be competing for overlapping epitopes on VP2. Additional anti-IPNV monoclonal antibodies can be purchased from DiagXotics that are not neutralising; this may result in a much more sensitive assay.

Initially high non-specific binding was a problem with the ELISA; this was reduced to a certain extent with the use of the low salt wash buffer (containing Tween) rather than PBS for dilution of IPNV, polyclonal antibody and conjugate. However it was not until caesin was used for blocking and added to the low salt wash buffer diluent that non-specific binding was reduced to below an O.D. of 0.1.

Unfortunately due to time constraints the specificity of the ELISA could not be evaluated. However the specificity of the polyclonal antiserum to IPNV serotypes found in the UK is known from neutralisation titrations, it was shown to only neutralise A2 (Sp.) serotype. There may still be some cross reactivity in ELISA with A3 (Ab.) serotype particularly since the AS-1 monoclonal is specific to VP2 and will react with all serotypes of IPNV in serogroup A.

The ELISA has potential for use as a confirmatory test after cell culture has identified a viral infection and eliminates the need for a titration neutralisation unless the serotype of the virus is necessary. The ELISA has the advantage of being very rapid with results obtained in approximately 5 hours, rather than 7 days. Recently RT-PCR and ELISA have been combined as a diagnostic technique for IPNV (Munoz, Opazo, Burzio and Miguel, 2000). They established that the RT-PCR-ELISA is more sensitive than RT-

PCR alone but did not quote sensitivities; this method has potential as an alternative diagnostic technique.

The immunohistochemical staining of IPNV in pancreatic lesions was successful using the AS-1 monoclonal. The sensitivity of the assay could not be assessed, however Biering and Bergh (1996) estimated the sensitivity of their immunohistochemistry (IHC) to be 10^5 $TCID_{50}$/ml based on infection trials with larval halibut. It is likely that the assay would be most useful for confirmation of IPNV infection where pathology is apparent. As a diagnostic method it allows for the simultaneous evaluation of morphological lesions and IPNV staining (Evensen and Rimstad, 1990). It is also a very rapid, straightforward test with results visible in 4-5 hours. It is possible that use of the kidney for immunohistochemistry may be more sensitive since virus is isolated from the kidney, often regardless of any attendant pathology in the pancreas. It has been noted that in formalin fixed tissue antigens may be masked by cross-linking of proteins around the antigenic sites (Elias, Gown, Nakamora, Wilbur, Herman, Jaffe, Battifora and Brigati, 1989). Staining may be further increased with the use of antigen retrieval-IHC, microwaving in the presence of a suitable buffer to avoid tissue degradation (Maniatis, Morris, Adams and Pearson, 2000). An advantage to using the AS-1 monoclonal is that as it is specific to VP2 it will stain all serotypes of IPNV.

A certain amount of background staining was visible in the IPNV positive tissue stained with PBS and the IPNV negative tissue stained with AS-1 monoclonal, however there were still marked differences and IPNV positive staining was clearly visible. There may be potential for this to be improved in the future.

Pathology for IPN not only includes pancreatic lesions but sloughing of the gut mucosa to form a catarrhal exudate (Smail *et al.* 1995). Smail *et al.* (1995) demonstrated the immunohistochemical staining of the necrotised tips of the gut villi in Atlantic salmon suffering an acute outbreak of IPN. Interestingly in this study whilst the pancreatic lesions stained positive for IPNV, the extensive gut pathology remained unstained.

In-situ hybridisation may be a more sensitive diagnostic method for IPNV and could be examined in the future. Biering and Bergh (1996) developed *in-situ* hybridisation for IPNV in halibut larvae and found that it was no more sensitive than the IHC, but suggested that *in situ* hybridisation may be amplified either by using *in situ* PCR or using amplifying complexes between the probe and the antibody.

In conclusion from the methods presented in this chapter, RT-PCR, ELISA and IHC, RT-PCR was found to be the most sensitive in tissue homogenates. The ELISA is in need of further validation and sensitivity may be improved with the use of an alternative monoclonal antibody raised against a different IPNV protein. Despite the insensitivity of the RT-PCR and ELISA they are both very rapid and if used in conjunction with cell culture provide conclusive results within one day. IHC has the advantage of allowing simultaneous evaluation of lesions and the presence of IPNV in clinical cases. Cell culture remains the most sensitive method of detection for IPNV.

6. Final Discussion

Microsatellites are ideally suited to disease resistance studies, particularly those involving IPNV since laboratory based challenges are rarely successful in producing clinical disease (Sadasiv, 1995, Taksdal *et al.*, 1998). The use of microsatellites allows field trials to be carried out; the results of which are likely to be a much more accurate representation of differences in resistance to infection between families than that of a laboratory based challenge.

Many problems were however encountered in the microsatellite study, mainly due to the lack of precautions taken by the fish farm to prevent the mixing of family fish with production fish whose parents had not been genotyped. The project was possibly a little ambitious bearing in mind the early stage of the family programme at the fish farm. However the microsatellite system was carefully examined as a result of this to ensure that the mistakes were not within the genotyping system with the result that it should reliably assign approximately 80% of fish susceptible to disease to a single family in the future. Due to the problems experienced with the microsatellite study and the amount of time taken to process the data, samples were not taken from further years' mortalities in the field. As a result the original aim of comparing data from the field to that obtained from experimental challenges could not be fulfilled.

A succession of laboratory based IPN challenges were undertaken in this study. These however did not produce clinical IPN and indeed initially many of the fish did not even become carriers but appeared to have completely cleared the virus. This may have been as a result of an alteration of the virus during cell culture (Rimstad *et al.*, 1991), the isolate may not have been particularly virulent or alternatively the virulence may have

been lost during storage. The use of a different isolate of IPNV stored for an equal length of time appeared to persist in the fish and actively replicate but still did not cause clinical disease. It is remarkable that a virus that appears to be so pathogenic in the field can replicate to titres as high as 10^{11} $TCID_{50}$ /g kidney in fish in experimental facilities and not cause death or even IPN pathology. Wolf (1988) states that where IPNV is the cause of death one finds titres of between 10^6 to 10^9 infection units per gram of tissue. It is likely that in the field titres of this level would cause death however it is clear that the statement does not apply to Atlantic salmon post-smolts infected with IPNV under experimental conditions.

The reasons for the discrepancy between the field and laboratory situation are unclear but it would seem that there are aspects to the disease in the field that are not replicated in the laboratory. Not in the least of which is the very slow progression of the disease over three months, something not usually associated with viral diseases or mirrored under experimental conditions. By chance this was the case with the control fish that were later proven to be IPNV positive yet still mortality was not demonstrated. The dynamics of the disease in the field are poorly understood and further work is necessary to elucidate this.

Landcatch maintain up to 200 separate family groups of Atlantic salmon until the fish are approximately 8 months old, at this time the fish are mixed and placed in large tanks to make room for the next years batch of eggs. Since the microsatellite study had given no indication as to which families were susceptible or resistant, the families used in the experimental challenge were chosen at random. Data gained from this challenge may have been more informative had an indication of resistance been known.

Still marked differences were found between different families of Atlantic salmon in their ability to respond to and clear IPNV. Certain families exhibited very high titres of the virus (10^{11} $TCID_{50}$ /g kidney) whilst others had very low titres (10^{2} $TCID_{50}$ /g kidney) due to the fact that certain individuals had cleared the virus altogether at 3 weeks post injection. These differences are likely to be genetic since all the fish were injected and sampled on the same day and suggest that there is potential for improvement in resistance to IPN in Atlantic salmon that should be explored in the future.

Atlantic salmon smolts about to be put to sea are more often than not vaccinated with a triple vaccine against *Aeromonas salmonicida*, *Vibrio anguilarum* and *Vibrio salmonicida* 6 weeks prior to seawater transfer. The family fish used in this study were vaccinated against *Aeromonas salmonicida* only two weeks prior to transfer due to an error at the fish farm. The time of experimental IPN challenge was delayed to 4 weeks post transfer as a result. This delay may have affected the success of the challenge, since in previous challenges the fish were challenged within 48 hours post transfer. This may also explain the Vibriosis mortalities experienced during the family challenge as only the vaccine against *Aeromonas salmonicida* was administered not the triple vaccine as planned.

Challenges with IPNV in Atlantic salmon fry have previously been successful (Taksdal *et al.*, 1997, Ramstad *et al.*, 1997). It is possible that the pattern of mortalities experienced with challenging different families of Atlantic salmon fry may be reproduced upon challenge with post-smolts. This requires the use of the same family

groups, with the post-smolt challenge using the siblings of the fry. This is hampered by the lack of an experimental challenge model for IPNV in post-smolts. However it could be attempted in the future either with the use of an experimental challenge should one become available or using a field challenge aided by the use of microsatellites. Should there be a good correlation between the two for the purposes of disease resistance trials the problematic challenges with post-smolts would not be needed and could be replaced with a fry challenge alone.

It was found that neutralising antibody titre did not correlate to virus titre, this is in agreement with Reno (1976), Smail and Munro (1985), and Biering (1997). It is possible that neutralising antibodies only play a limited role in defence against IPNV in a natural infection, based on the statistically insignificant differences in neutralising antibody titres throughout the course of the progressing natural IPNV infection in the control fish. Lysozyme was also found not to correlate well with IPNV titre. This may have arisen due to the concurrent bacterial infection with *Vibrio* spp. that was not a problem for all fish. However the potential role of lysozyme in defence against IPNV has been demonstrated (Siwicki *et al.,* 1998) and this should be investigated further along with phagocytic cellular responses.

Many studies have been carried out into lysozyme activity (Mock and Peters, 1990, Roed *et al.,* 1993, Fevolden *et al.,* 1994, Marsden *et al.,* 1996). The different methods used to measure lysozyme and expression of results, e.g. units/ml or units/g tissue allows for little comparison between studies. It is suggested that assays to measure not only lysozyme but also other parameters be standardised to aid comparison.

During the course of the family challenge with IPNV the control fish were found to be IPNV positive despite the fact that the fish had been found negative prior to the challenge. Exactly how the fish became infected was not proven but it highlighted the need for highly sensitive diagnostic methods. This has previously been noted with many pathogens but particularly IPNV (Dixon and Hill, 1983, Lopez-Lastra *et al.*, 1994, Bruneau *et al.*, 1999). Various diagnostic methods were investigated in this study and tissue culture was found to be the most sensitive.

RT-PCR is perhaps the test with the greatest potential for high sensitivity and functions well as a test to confirm IPNV infection once CPE is present in cell culture. However due to the high concentration of nucleic acids in the kidney amplification from tissue samples it was problematic. The RNA tissue extraction kit was not able to remove all high molecular weight DNA, protein and RNA. The most efficient extraction method was Tri reagent, yielding only RNA, however due to the high concentration of RNA in the kidney the RT-PCR was found to be far less sensitive than cell culture. Nevertheless it may be possible to increase the sensitivity of the test with the treatment of extracted kidney and IPNV RNA with RNase A to remove ssRNA from the kidney (Nicholson, 1996).

An ELISA test was developed with a sensitivity of $10^{12.5}$ $TCID_{50}$/ml, due to time constraints the ELISA could not be fully evaluated. The results did however indicate that it may function more efficiently if an alternative monoclonal antibody was chosen. Immunohistochemistry was shown to be a useful rapid diagnostic test, it allows the simultaneous evaluation of pathology and IPNV infection (Evensen and Rimstad, 1990) however is unlikely to be very sensitive.

This study has evaluated the use of microsatellite analysis to genotype fish susceptible to IPNV and discussed the problems associated with it. Various diagnostic methods have been developed with a view to increasing the detection limit of IPNV, however cell culture remains the most sensitive method. This work has also provided a valuable insight into the responses of different families of Atlantic salmon to infection with IPNV under experimental conditions.

Disease resistant fish are the ideal solution to many disease problems. However this is a very long term goal and requires years of preparation and commitment particularly if disease resistance at the family level rather than strain is the goal. It also requires trial periods to iron out any unforeseen problems and a large amount of investment. Few fish farms are in a position to carry out this type of work or have the necessary experience. However it is a goal worth pursuing, particularly with IPN in light of the huge financial losses and the lack of either control methods or a suitable vaccine.

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Appendix 1

Buffers and Media

DNA Extraction Buffer

200mM Tris-HCl, pH 8.0

250mM NaCl

25mM EDTA

0.5% SDS

Dissolve tris, NaCl and EDTA into nanopure water and autoclave. SDS was added after autoclaving from a 25% stock solution that had previously been filter sterilised.

TE Buffer

0.1mM EDTA

10mM Tris

Dissolve both in 100ml nanopure water and alter pH to 8.0 with dilute HCl, autoclave.

TBE Buffer (10x)

55g Boric acid

108g Tris

8.3g EDTA

Dissolve into nanopure water and filter using 0.45µm filter. Dilute 1:10 with nanopure water for use.

TNE Buffer

100mM NaCl

20mM Trizma

1mM EDTA

Dissolve all 3 into nanopure water, adjust to pH 7.5, and autoclave.

10% APS

0.1g ammonium persulphate

1ml nanopure water.

Dissolve.

ABI loading dye - 25mM EDTA/50mg/ml blue dextran

0.93g EDTA

Add 90ml nanopure water, adjust to pH8

Bring to 100ml with nanopure water.

Add 50mg blue dextran to 1ml of EDTA solution.

Tryptone soya agar+sodium chloride

20g TSA

7.5g sodium chloride

Mix and autoclave, pour when hand hot into 20 petri dishes.

10% Neutral Buffered Formalin

4g sodium dihydrogen phosphate (monohydrate)

6.5g disodium hydrogen phosphate (anhydrous)

100ml formaldehyde

900ml distilled water

1% Crystal Violet (500 ml)

Dissolve 5g crystal violet into 100 ml 95% ethanol. Make up to 500 ml with PBS.

Dissolve and filter.

Safranine Solution

0.25g safranine

10ml 95% ethanol

90ml dH_2O

Iodine Solution

1g iodine

2g potassium iodide

300ml dH_2O

Scotts tap water substitute

3.5g sodium bicarbonate

20g magnesium sulphate

1l tap water

Dissolve by heating if necessary, add several crystals of thymol to preserve.

Mayers Haemalum

2g haematoxylin

0.4g sodium iodate

100g potassium alum

2g citric acid

100g chloral hydrate

2l distilled water

Allow the haematoxylin, alum and sodium iodate to dissolve overnight in 2l of distilled water. Add chloral hydrate and citric acid, bring to the boil for 5 minutes.

1% Eosin

20g eosin yellowish

Dissolve in 500ml distilled water, top up to 2l with distilled water.

Putts Eosin

4g eosin yellowish

2g potassium dichromate

40ml saturated Aq picric acid

40ml absolute ethanol

320ml distilled water

Dissolve eosin and dichromate in the ethanol. Add distilled water then picric acid.

For final eosin solution

Mix 1 part Putts eosin with 8 parts 1% eosin.

Sodium Phosphate Buffer pH 6.2 (0.07M)

1. 5.5g sodium di-hydrogen orthophosphate in 200 ml dH_2O

2. 2.83g di-sodium hydrogen orthophosphate in 100 ml dH_2O

Take 122.1 ml of solution 1 and mix with 27.0 ml of solution 2. Add 276 ml dH_2O and autoclave.

Tris Buffered Saline (TBS) pH 7.6

50 mM Tris-HCl

123 mM NaCl

ELISA Buffers

Chapter 4 ELISA

Carbonate Buffer (pH 9.5)

1.5g/l sodium carbonate

2.93g/l sodium bicarbonate

0.2g/l sodium azide

PBS Thiomersol (pH 7.2)

8.0g/l sodium chloride

0.2g/l potassium di-hydrogen orthophosphate

1.4g/l disodium hydrogen orthophosphate

0.2g/l potassium chloride

0.2g/l thiomersol

PBST (pH 7.2)

PBS thiomersol with 0.5 ml Tween 20/litre

PBSN (pH 7.2)

PBS thiomersol with 1 % sodium azide.

Chapter 5 ELISA

Coating Buffer

Dissolve one carbonate-bicarbonate capsule (Sigma) into 100ml dH_2O.

Low Salt Wash Buffer (10x)

24.2g trizma base

222.2g sodium chloride

1g thiomersol

5 ml Tween 20

Dissolve in 1 litre of dH_2O and adjust pH to 7.3 with concentrated HCl.

High Salt Wash Buffer (10x)

24.2g trizma base

292.2g sodium chloride

1g thiomersol

10 ml Tween 20

Dissolve in 1 litre of dH_2O and adjust pH to 7.7 with concentrated HCl.

Phosphate Buffered Saline (PBS)

(0.02M phosphate, 0.15M NaCl)

0.876g sodium di-hydrogen orthophosphate

2.56g di-sodium hydrogen orthophosphate

8.77g sodium chloride

Dissolve in 1 litre dH_2O and adjust to pH 7.2.

Substrate Buffer

21g citric acid

8.2g sodium acetate

Adjust pH to 5.4.

Appendix 2

Histology Methodology

Processing Schedule

1. 50 % methylated spirit	1 hour
2. 80% methylated spirit	2 hours
3. 100% methylated spirit	2 hours
4. 100% methylated spirit	2 hours
5. 100% methylated spirit	2 hours
6. 100% ethanol	2 hours
7. 100% ethanol	2 hours
8. chloroform	1 hour
9. chloroform	1 hour
10. molten wax	2 hours
11. molten wax	2 hours
12. molten wax	2 hours

Haematoxylin and Eosin staining

1. Xylene (dewaxing)	5 min
2. Absolute alcohol 1	2 min
3. Methylated spirit	1.5 min
4. Wash in tap water	
5. Haematoxylin	5min
6. Wash in tap water	
7. Acid alcohol	3 quick dips
8. Wash in tap water	
9. Schotts tap water	1 min
10. Wash in tap water	
11. Eosin	5 min
12. Wash in tap water	
13. Methylated spirit	30 sec
14. Absolute Alcohol II	2 min
15. Absolute alcohol I	1.5 min
16. Xylene (clearing)	5 min
17. Xylene (coverslip)	

Appendix 3

Genotyping results and allele coding

Table A-1: Results of Out Skerries mortalities parentage assignment

Family	Number of susceptible individuals assigned to family
2	2
4	2
14	2
19	4
29	3
67	2
69	4
71	3
72	2
74	5
75	2
76	2
81	2
94	4
102	3
113	4
150	3
152	2

Other families with only one offspring assigned to them:

1, 16, 23, 28, 42, 48, 50, 51, 56, 66, 70, 73, 77, 89, 90, 93, 95, 96, 100, 108, 116, 117, 122, 147, 153, 162, 163.

Allele Assignment

Table A-2: Allele coding for each locus.

Assigned letter	Ssa202	Ssa171	Ssa197	Ssa85
A	240	200	164	123
B	244	212	168	125
C	248	216	172	131
D	252	220	176	133
E	256	224	180	135
F	260	228	184	136
G	264	232	188	138
H	268	236	192	140
I	272	238	196	145
J	276	245	200	147
K		250	204	149
L		205	208	151
M		218	212	155
N		240	220	157
O		222	244	159
P			252	142
Q			256	
R			260	
S			264	

Double underline indicates alleles in 1996 year class only

Single underline indicates alleles in 1997 year class only

Appendix 4

Trends in temperature for each challenge at Aultbea

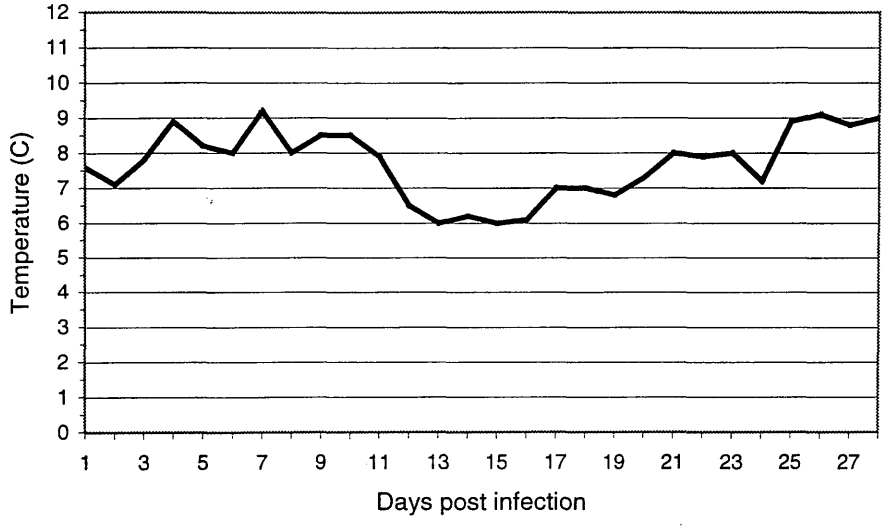

Figure A-1: Seawater temperature trends during Passage 1 at Aultbea.

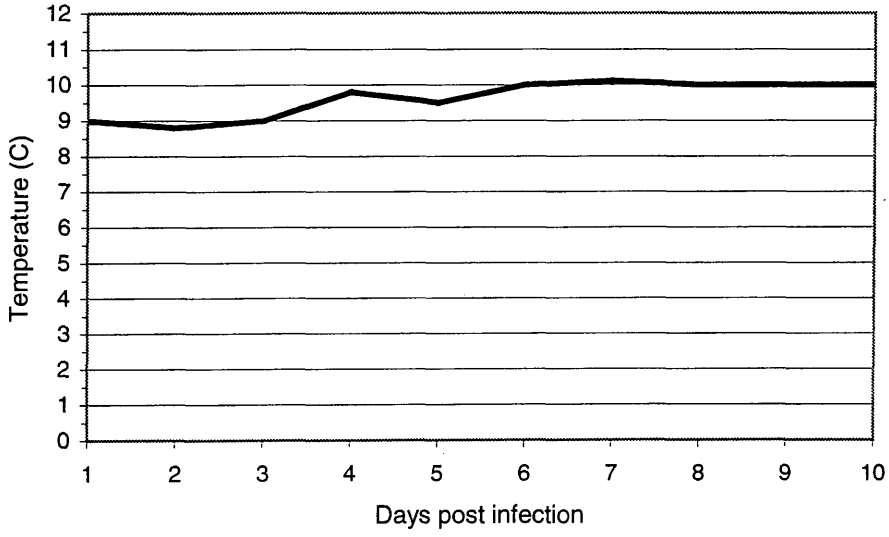

Figure A-2: Seawater temperature trends during Passage 2 at Aultbea.

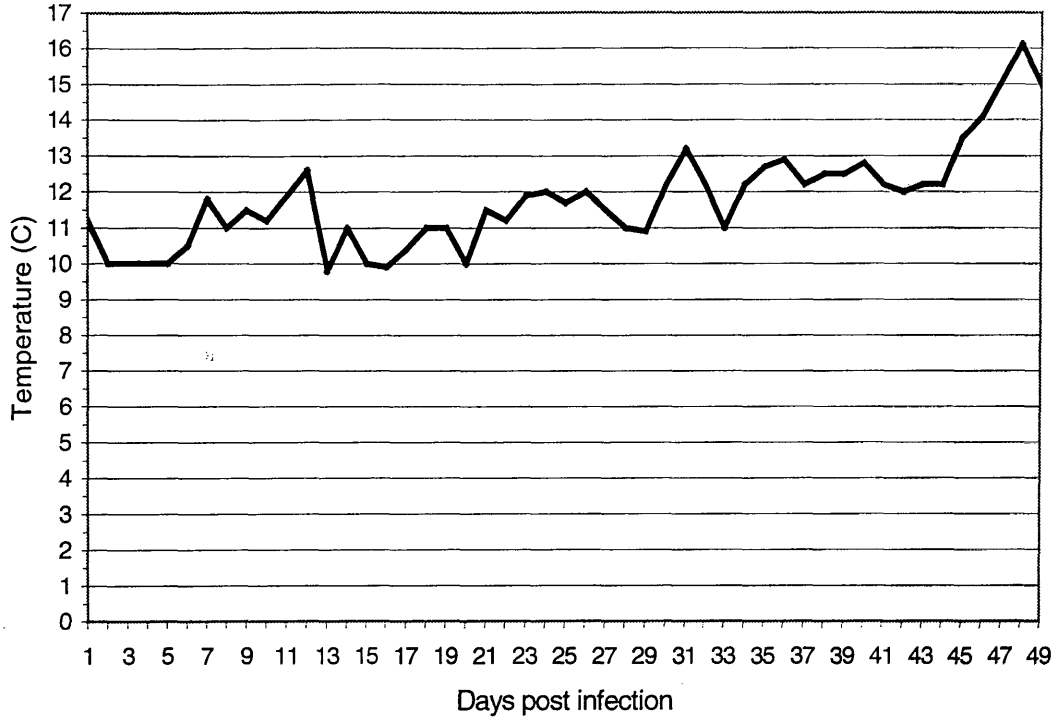

Figure A-3: Seawater temperatures during the family challenge.