Thesis submitted for the degree of

Doctor in Philosophy

The innate immune response of Atlantic salmon head kidney macrophages to Infectious Pancreatic Necrosis Virus (IPNV)

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Declaration

I, Gavin McKinley hereby declare that the work and results presented in this thesis were carried out by me at the Institute of Aquaculture, University of Stirling, Scotland. The work presented in this thesis has not previously been submitted for any other degree or qualification. All information from other sources has been acknowledged.

Gavin McKinley

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Psalm 121.

Abstract

Infectious pancreatic necrosis virus (IPNV) is the aetiological agent of infectious pancreatic necrosis (IPN), a disease associated with serious economic loss in Atlantic salmon (*Salmo salar*). The interaction between IPNV and the host is poorly characterised. IPNV has been detected within macrophages in natural and experimental infections. The macrophage is an important component of the host immune system, participating in innate and adaptive immune responses. The overarching objective of this project was to study aspects of the interaction between IPNV and innate immune responses in the Atlantic salmon macrophage.

Methods were developed for the isolation and *in vitro* culture of Atlantic salmon macrophages. These cells were isolated from head kidney using percoll gradients and subsequently cultured in 24 well plates using Leibovitz L-15 medium containing penicillin, streptomycin and foetal calf serum. This procedure enabled the *in vitro* culture of macrophages for 9 days post isolation. Real time RT-PCR assays were developed to quantitate the expression of IPNV, Interferon (IFN), Mx, and Elongation factor 1 (ELF-1) in IPNV-infected macrophages and uninfected controls. ELF-1 is utilised as a control gene for relative quantitation in RT-PCR studies. The RT-PCR assays utilised target-specific primers, and MGB probes. Assay efficiencies varied from 0.85 to 0.99, these were suitable for quantitative RT-PCR analyses.

IPNV was demonstrated to replicate in macrophages cultured *in vitro* as assessed by quantitative RT-PCR. IPNV levels in macrophages were greatest at the early stages of infection. Virus was detected in infected macrophages throughout the nine day period of investigation. Quantitative RT-PCR analyses of the expression of the immune response

genes IFN and Mx suggested that IPNV blocks IFN production, as opposed to blocking IFN signalling.

The ability of three immunostimulants, Lipopolysaccharide (LPS), macrophage activating factor (MAF), and glucan to up regulate immune responses in IPNV-infected macrophages was also investigated. None of these immunostimulants were able to enhance expression of IFN and Mx, suggesting that these substances may not represent useful therapeutic means of mitigating IPN in Atlantic salmon.

List of Abbreviations

2ME	: 2 mercaptoethanol
ANOVA	: analysis of variance
CHSE-214	: Chinook salmon embryo (cell line)
CFS	: guinbuna crucian carp cell line
Con A	: concanavalin A
cDNA	: complementary deoxyribonucleic acid
CPE	: cytopathic effect
Ct	: threshold cycle
DEPC	: diethylpyrocarbonate
DNA	: deoxyribonucleic acid
dsRNA	: double stranded RNA
ELISA	: enzyme-linked immunosorbent assay
ELF-1	: elongation factor 1
EMEM	: minimal essential medium with Earle's salts
FCS	: foetal calf serum
IFN	: interferon
IL	: interleukin
IPNV	: infectious pancreatic necrosis virus
i.p.	: intraperitoneal
kDa	: kilodalton
l	: litre
L-15	: Leibovitz-15
LPS	: lipopolysaccharide
M	: molar
MAF	: macrophage activating factor
mRNA	: messenger RNA
mM	: millimolar
min	: minute

MOI	: multiplicity of infection
NBT	: nitroblue tetrazolium
NEAA	: non-essential amino acids
PBS	: phosphate buffered saline
PCR	: polymerase chain reaction
PMA	: phorbol myristate acetate
Poly I:C	: Polyinosinic-polycytidylic acid
qRT-PCR	: quantitative reverse-transcription PCR
RNA	: ribonucleic acid
RT	: reverse transcription
RT-PCR	: reverse-transcription PCR
RTG	: rainbow trout gonad cells
SD	: standard deviation
TBS	: Tris buffered saline
$TCID_{50}$	: 50% tissue culture infective dose
TLR	: toll like receptor
TNF-α	: tumour necrosis factor alpha

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Chapter 1 - Literature review

1.1 Infectious Pancreatic Necrosis Virus

Infectious pancreatic necrosis virus (IPNV) is the etiological agent of a highly infectious disease of wild and cultured fin fish (Hill and Way, 1995). IPNV is the type species of the genus Aquabirnavirus of the family birnaviridae (Dobos *et al.*, 1979). Aquatic birnaviruses generally have a wide host range and both clinical disease and carrier states have been reported in a variety of salmonids as well as non-salmonid fish world-wide (Wolf, 1988; Hill and Way, 1995). The classification of aquatic birnaviruses have been divided into two serogroups A and B based on serological analyses (Hill and Way, 1995). Nine different serotypes have been distinguished so far in serogroup A, whereas serogroup B contains only one serotype (Hill and Way, 1995; Song, 2005).

1.1.1 Structure

IPNV is an unenveloped icosahedral virus containing two segments of dsRNA (Dobos, 1995), which encode five proteins designated VP1 to VP5 (Weber *et al.*, 2001). Genome segment A of 3097bp, contains a large open reading frame (ORF) encoding a 106 kDa polyprotein which is cleaved to produce two structural polypeptides, pVP2 and VP3, and one non-structural polypeptide, NS (or VP4) (Ducan *et al.*, 1987). pVP2 is a precursor of the major capsid protein VP2 (Blake *et al.,* 2001), which is further cleaved to yield VP2 during virus maturation. VP3 is thought to be an internal virion protein (Blake *et al.,* 2001), whereas VP2, being the outermost protein, is involved in attachment to cells

(Sadasiv, 1995). Genome segment A contains an additional small open reading frame (ORF) which overlaps the amino terminal of the polyprotein ORF and is translated in a different reading frame; this ORF encodes a 17-kDa arginine rich minor polypeptide (Magyar *et al.,* 1998) designated VP5 (Weber *et al.,* 2001). The smaller genome segment B (2784 bp) is monocistronic and encodes VP1 (94 kDa), the putative virion-associated RNA-dependent RNA polymerase (Blake *et al.,* 2001). VP1 is present in the virion in two forms, as a free polypeptide (VP1) and as a genome linked protein (VPg). (Calvert *et al.,* 1991).

1.2 Antigenic composition

It has been demonstrated that IPNV serotype varies between broad geographical areas (Melby *et al.,* 1994). Whilst the vast majority of isolates have been found to be antigenically-related to the original reference serotypes (VR299, Sp and Ab) of IPNV, (Hill and Way, 1995) found there to be a high degree of antigenic diversity amongst isolates with some relating only relatively weakly with the three traditional serotypes. A standardized serological classification scheme was proposed by (Hill and Way, 1995) for serotyping IPNV and the other aquatic birnaviruses, through which they divided the birnaviruses into serogroups A and B. Serogroup B consists of a single serotype (B1), whilst serogroup A has been divided into nine serotypes A1-A9. Serological characterization of this group of viruses is important both for epizootiological reasons as well as the development of vaccines (Reno, 1999). According to (Frost and Ness, 1997), in the northern part of Europe, aquatic birnaviruses that cause clinical infectious pancreatic

necrosis (IPN) in Atlantic salmon show a surprisingly homogeneous nature, all belonging to the Sp serotype of serogroup A. From the European serotypes Sp is usually highly virulent whereas Ab displays a low virulence (Dorson, 1988).

1.3 Clinical signs of IPNV-induced disease

In salmonid hatchery populations, a sudden and usually progressive increase in mortalities, particularly in faster growing individuals, is often the first sign of an outbreak (Hill, 1982). Mortality can be variable, ranging from negligible to almost 100% in extreme cases (Taksdal, 1999). IPN commonly results in mortality that is inversely proportional to the age of the fish, being typically highest in the youngest fish and relatively rare in older fish, in which infections are often asymptomatic (Wolf, 1988). Clinical signs typically appear on day 3 to 5 (fry) or on day 8 to 10 (fingerlings) after exposure to the virus. Peak mortalities usually occur between days 12 to 18 (Noga, 2000), however Atlantic salmon smolts can suffer from the disease shortly after transfer to seawater (Bowden *et al*., 2002). The time course of clinical disease varies with fish age, species, temperature and other conditions (Wolf, 1988). Larger, faster growing young fish, as a rule, are the first to die from IPN (Hill, 1982, Post, 1987). Mortality develops rapidly at about 10-14°C, is protracted at lower temperatures, and can be reduced at higher temperatures (Ahne et al., 1989).

1.3.1 External signs

In a fish farm which has never previously been affected by an outbreak of IPN the fundamental sign of the disease is the occurrence of high mortality in young fry during the first two months after coming onto feed (Roberts and Shepherd, 1997). Infected fish show a gradual loss of equilibrium with a tendency to swim on their sides or in spirals in a corkscrew fashion (Dorson, 1988). Prior to death, fish may become lethargic with violent flexing of the body, suggesting abdominal distress (Candan, 2002; Roberts and Shepherd 1997). IPNV-infected fish frequently exhibit an overall darkening of the body (Post, 1987 Wolf, 1988; Roberts and Shepherd, 1997) and swelling of the ventral region (Dorson, 1988; Wolf 1988). Haemorrhages are sometimes present in ventral areas, including the ventral fins (Wolf, 1988; Ahne *et al*., 1989). Many victims trail long, thin, whitish, castlike excretions from the vent (Wolf, 1988). According to Roberts and Shepherd (1997), after early mortalities in an outbreak showing the acute disease, the picture changes to a chronic from where the mortalities are lower and fish take longer to die. In this form, the fish appear quite black and show severe exophthalmia. They are very anaemic due to the severe internal bleeding and this particularly evident in the gills.

1.3.2 Internal signs

As suggested by its name IPNV-infection produces marked pancreatic necrosis (Dorson, 1988), however histopathological changes may also occur in adjacent adipose tissue, in renal hematopoietic tissue, in the gut and in the liver (Wolf, 1988). IPN virus replicates largely within pancreatic acini and causes pyknosis and karyorrhexis with a moderate

inflammatory infiltrate (Ahne *et al*., 1989; Ferguson, 1989). Microscopically, there is focal coagulative necrosis of the acinar and islet cells of the pancreas and of the haemopoietic cells of the kidney (Reno, 1999). Prominent gut enteritis with sloughing of the mucosa has been reported as a common characteristic (Ahne *et al*., 1989; Ferguson, 1989; Smail *et al*., 1995). Smail *et al*., (1995) report that this produces a catarrhal exudate which they suggest inhibits digestion and leads to malabsorption of the gut contents and the normal passage of food through the gut. The development of a catarrhal exudate is also reported by Noga, (2000), which may help to explain why the digestive tracts of infected fry are almost always devoid of food (Wolf, 1988). Prior to 1940, the disease was called acute catarrhal enteritis because of the typical opalescent mucus plug characteristically found in the intestine of obvious cases of the disease (Post, 1987). In chronic form, affected fish are very anaemic due to severe internal bleeding and this is shown especially in the gills and liver; the swim bladder and kidney may be enlarged or the entire abdomen filled with fluid, which produces the swollen dropsical external appearance (Roberts and Shepherd, 1997).

1.4 Host and geographic range

The first isolation of IPNV was made from brook trout (*Salvelnius fontinalis*) in 1957 in the United States. This prototype isolate was deposited with the American Type Culture Collection (ATCC) in 1963 and given the reference number VR299. For several years thereafter all isolates which were neutralized by antiserum against the VR299 reference virus were referred to as IPNV strains (Hill and Way, 1995). For several years after it was

first discovered, IPNV was known only from North America and only as a pathogen that produced disease and mortality in trout fry, however the movement of fish and especially eggs, in the international market is thought to be one of the main reasons for the dissemination of the virus worldwide (Reno, 1999). With improvements in virus detection methods, it is apparent that IPNV is quite ubiquitous (Sadasiv, 1995). There are however some countries that are reported to be free of IPNV, these being Australia (Wolf, 1988; Reno, 1999; Taksdal, 1999), Iceland (Reno, 1999; Taksdal, 1999), Sweden (Ariel and Olesen, 2002; Murray *et al*., 2003) and New Zealand (Wolf, 1988 and Taksdal, 1999). The most susceptible species of fish appear to be rainbow trout (*0ncorhynchus mykiss*), brook trout, brown trout (*Salmo trutta*) and Atlantic salmon (Kent and Poppe, 1988; Wolf, 1988; Moya *et al.,* 2000). While freshwater salmonids are the group most commonly afflicted with clinical IPN (Noga, 2000). IPNV has been isolated from many non salmonid species of fishes and shellfish (Stoskopf, 1993; Sadasiv, 1995). In most cases, these isolates have not been proven to be pathogenic for the host species (Stoskopf, 1993; Sadasiv, 1995; Cutrin *et al.,* 2000), although they have been pathogenic to trout. Thus at present, according to Noga (2000) these aquatic species are most clinically important in acting as nonsusceptible viral reservoirs. Ahne *et al*., (1989) have demonstrated one such example of this by showing that carnivorous fish such as pike (*Esox lucius*) can become infected with IPNV by feeding on infected food fish, and as they develop IPNV infection cycles, which in turn leads to carrier status.

1.5 Vectors of IPNV

Mcallister and Owens (1992) demonstrated that wild piscivorous birds, resident in salmonid fish hatcheries can be contaminated with IPN virus under natural conditions and can potentially serve as mechanical vectors for the dissemination of the virus. Mortensen, (1993) studied the passage of IPNV through a marine food chain, including uptake and release of the virus by bivalve molluscs, further transmission to prawns, and from prawns to trout. Mortensen's findings showed that IPNV could be detected in the faeces of contaminated scallops, and the virus was then able to be transmitted to prawns that ingested either dead scallops, infected faeces or pseudofaeces. These results suggest that viral transmission may occur between aquatic invertebrates and fish. Smail *et al*., 1993(a) have demonstrated the ability of IPN to survive in commercial fish silage for periods of many days, especially at low temperatures. It was the opinion of Smail *et al*., 1993(b) reported that if fish silage is not treated to inactivate IPNV, it could lead to the spread of IPNV via application to pasture and permit subsequent return of the virus to fresh water sources via the water cycle.

1.6 Transmission

IPN only occurs through interaction between susceptible fish and virus. Salmonid immune defence systems are either not present or are overcome (Sadasiv, 1995). Each cell infected with virus can produce up to 1000 new infectious particles within a few hours, depending on the temperature and cell type (Dobos, 1995). IPNV has been shown to be able to

transfer both vertically from parent fish to offspring and horizontally via water (Lopez-Lastra *et al*., 1994). Anderson (1982) has suggested that information about the mechanisms and patterns of pathogen transmission from infected to susceptible individuals can be used to develop methods for the prevention and control of infectious disease outbreaks.

1.6.1 Horizontal transmission

IPN is a contagious disease (Taksdal *et al*., 1998). IPNV shed in infected faeces will possibly contaminate surrounding areas and lead to horizontal transmission (Wolf, 1988). Faecal pseudocasts are considered as major sources of the virus by Noga (2000) who states that during epidemics, IPNV is readily transmitted horizontally by contact and ingestion of infected tissue. Studies conducted by Bebak *et al*., (1998) demonstrated that rainbow trout infected with IPNV can begin excreting virus within two days after infection, and infected fish that are shedding the virus can infect others in the population within another two days. They found that it was possible for 75% of the population to become infected in less than a week after initial shedding of the virus began. Mcallister and Bebak, (1997) sampled the discharge and downstream distribution of IPNV from three fish hatcheries and found the virus could be detected for at least 19.3km below the point of effluent discharge. However based on IPNV prevalence within the surrounding streams it would appear that chronic low level exposure to IPNV in the stream water did not appear to pose a significant risk to resident salmonid and non-salmonid fish. It was concluded that population density is associated with transmission and the probability of infection or an

epizootic, and that the population density within the sample streams was below the threshold needed for infection.

1.6.2 Vertical transmission

IPNV persists in clinically diseased fish as well as in asymptomatic carriers which shed high levels of virus via infected reproductive fluids (Wolf, 1988; Lopez-Lastra, 1994; Sadasiv, 1995). IPNV can replicate in hosts for long periods without causing clinical disease, for this reason broodstock carriage has been considered a likely source of the virus for the lethal infection of progeny fish (Sadasiv, 1995). The hardened eggs of rainbow trout and artic charr (*Salvelinus alpinus*) were shown by (Ahne and Negele, 1985) to provide a surface for IPNV to adhere to because of the lobed and porous nature of their surface, which is in contrast to the chorion of eggs before hardening. Thus, the surface of the hardened eggs may provide anchorage for the virus and protect it from flowing water.

1.7 Entry of virus into fish

In horizontal transmission, it is likely that the digestive tract is the prime site of infection but the gills may also be important because the fry and fingerlings are easily infected by bath or immersion (Wolf, 1988). According to Ferguson (1989) the natural route for initial infection in the commercial hatchery is likely to be via water and therefore at the gill surface, infection may also take place via ingestion of virus contaminated faeces. In studies concerned with entry and sequential distribution of IPNV in turbot (*Scophthalmus maximus*) Novoa *et al*., (1995) noticed that after an immersion challenge the virus was

detected in skin mucus and intestine, therefore suggesting that these could be interpreted as portals of entry. However, virus could not be recovered from internal organs contesting this theory and suggesting that the skin and mucus act as potential barriers to infection. Swanson and Gillespie (1982) presented the first documentation of a viremia in IPNV infected fish, with the virus being detected in both the serum and the mononuclear cell fractions of the blood. Research has been performed to study the distribution and spread of the IPNV within infected fish (Swanson and Gillespie, 1982). After intraperitoneal (i.p.) infection of rainbow trout the virus replicated quickly in the pancreas and was also detected in the kidney and liver. *Nova et al*., (1995) studied the entry and sequential distribution of an aquatic birnavirus in turbot, and found the evolution of the viral titre in fish infected by i.p. injection showed that the kidney is a preferential replication site for birnavirus, since the highest titres were obtained in this organ.

1.8 Carrier status

The term carrier applies to survivors of IPNV infection that have no disease although high titres of virus can be isolated from their viscera (Reno, 1999). Whilst these fish no longer exhibit signs of the disease, they continue to shed the virus in their faeces, urine and reproductive fluids (Wolf, 1988; Lopez-Lastra *et al*., 1994). Many fish viral pathogens produce a persistent carrier state in the host; however little is known about the mechanisms involved in viral carrier states in fish and how the viruses evade the hosts defences (Ellis, 2001).

1.8.1 Sites of IPNV persistence within host

It is the opinion of Johansen and Sommer (1995) that because the kidney, which is a haematopoietic organ in fish, is most often the organ in which tests successfully detect IPNV carriers, this would suggest that one or more types of leucocytes may harbour IPNV. Swanson and Gillespie (1982) intraperitoneally injected rainbow trout with IPNV and reported that the virus was transported by phagocytic cells to the kidney. Johansen and Sommer (1995) suggested that adherent salmon head kidney leucocytes have a major role in maintaining the IPNV carrier state in Atlantic salmon. IPNV was shown to persist in salmon head kidney macrophages for 9 days after *in vitro* infection without causing cytopathic effect (CPE); (Collet *et al.,* 2007). There are many other reports of detection of IPNV from carrier fish in leucocytes (Johansen and Sommer, 1995; Cutrin *et al.,* 2005; Munro *et al.,* 2004; Garcia *et al*., 2006), however whilst IPNV has been reported to be associated with leucocytes there is doubt wether the virus actually replicates in these cells (Nova, Figueras, Secombes 1996; Munro *et al.,* 2006). Yu *et al.,* (1982) reported that whilst rainbow trout leucocytes harbour IPNV, they do not contribute in large measure to the high titres of IPNV found in haematopoietic organs, and concluded that in these organs and at other sites, the bulk of IPNV replication may occur in other cell types.

1.8.2 Defective interfering particles

The production of defective interfering (DI) particles is a common consequence of virus infection of animals, particularly by RNA viruses (Cann, 2001). Structural analyses of DI viral genomes revealed that the majority of them are deletion mutants which originate

from the genome of parental viruses (Lancaster *et al.*, 1998). According to Cann (2001), the presence of DI particles can profoundly influence the course and the outcome of a virus infection, and may result in a persistent infection by a virus that normally causes acute infection and is rapidly cleared from the body. DI particles may play a major role in determining the persistence of the carrier state for IPNV (Hill, 1982). Infectious hematopoietic necrosis virus (IHNV), a rhabovirus that produces an acute, lethal infection in rainbow trout is similar to IPNV in that fish surviving infections continue to harbour virus at subclinical levels. Subclinical persistence of virus in the tissues of IHNV survivors was first confirmed by Drolet *et al.*, (1995), in the form of truncated IHNV particles resembling rhabdovirus DI particles. Drolet *et al.*, (1995) suggested that these DI particles act as mediators of virus persistence, therefore providing a model for the maintenance of IHNV in salmon and trout populations. Kim *et al.,* (1999) conducted studies to determine if the truncated particles present in the tissues of survivor fish could interfere with viral replication. They were able to show that when explant tissue cultures were infected with purified IHNV, the liver tissues from survivor fish produced up to 10-fold less virus than control fish liver tissues. They were also able to demonstrate that only the supernatant media from cultured explants of survivor fish revealed truncated particles, whereas the control tissue supernatants contained normal virus particles. Hedrick and Fryer (1982) compared the persistence of IPNV in carrier brook trout and persistently infected cell lines and suggested that DI IPNV appeared to function in maintaining persistent infection. From the similarity between the interference observed *in vitro* and *in vivo*, Hedrick and Fryer (1982) suggested that DI virus production may also occur in carrier trout tissues. However it is the opinion of Kim *et al*., (1999) that although most viruses are thought to produce DI

particles in tissue culture cells and some of these DI particles have been shown to modulate infections in animals, much of the proposed impact of these particles in natural infections has been speculative.

1.9 IPN and post-smolt mortalities

According to Taksdal *et al*., (1995) a remarkable shift in the disease pattern of IPNV has occurred. In recent years, this disease has been associated with mortality in Atlantic salmon post-smolts about 8 weeks after seawater transfer (Smail *et al*., 1992; Jarp *et al*., 1992; Labus *et al*., 2001; Bowden *et al*., 2002). Post-smolt mortality is especially prevalent in Norway and the Shetland Islands (Bowden *et al*., 2002). Post-smolt mortality has caused great losses in recent years in Norwegian fish farms (Jarp *et al*., 1995; Eggset *et al*., 1997). In 1991, post-smolt mortality was at a high of 17% compared to normal mortalities of 3-5% of the previous years. Smail *et al*., (1992) were the first to show that IPNV of the Sp serotype was associated with mortality of post-smolts in Scotland. There have also been reports of smolt losses in the Faroe Islands (Smail *et al*., 1992). It has been suggested environmental and management factors may contribute to these mortalities (Smail *et al*., 1992; Jarp *et al*., 1995).

1.10 Influence of environmental stressors on the occurrence of IPN

According to Jarp *et al.*, (1996) it is likely that the IPNV is carried with the smolt to the sea site; however the causal mechanisms triggering outbreaks of IPN after seawater

transfer are not known. Jarp *et al*., (1995) demonstrated that an increased risk of IPN is associated with the age and location of seawater sites in Norway, with the risk of disease being significantly higher in new sites compared with older sites. It was suggested that IPNV might be transmitted to smolts, after being introduced to sea ongrowing sites, by a marine vector (Smail *et al*., 1992). However, Jarp *et al*., (1995) performed an epidemiological survey of IPN in post-smolts in Norway and demonstrated that clinical disease was associated with the combination of smolts, at the same seawater site from more than two hatcheries. Poor seawater adaptation at seawater transfer has been suggested to cause reduced resistance and especially increased susceptibility to IPN (Taksdal, 1999); however, studies by Jarp *et al.*, (1996) showed no relationship between the hypo-osmoregulatory capacity of Atlantic salmon, and the risk of clinical IPN after seawater transfer. According to Christie (1997), clinical IPN in Atlantic salmon occurs only under conditions of virus exposure, accompanied by additional stress factors including rise in temperature and or co-infections with other viruses. Jarp *et al*., (1995) suggested that the stress that the smolts are subjected to through transportation to seawater sites may result in an increased susceptibility to infection, which may explain high mortality in the first months after seawater transfer. According to Smail *et al*., (1995), 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.

1.11 Effect of IPNV on global aquaculture

Disease outbreaks remain a considerable obstacle to aquaculture production and development (Ariel and Olesen, 2002). Viral diseases cause very significant losses in aquaculture (Ellis, 2001), with IPNV being considered to be one of the most important diseases of farmed salmonids (Sadasiv, 1995). Atlantic salmon is economically the most important fish species farmed in Norway (Havarstein *et al.*, 1990). IPN causes large economic losses in Norwegian fish farming (Biering and Bergh, 1996). It is believed that more than 50% of the farmed Atlantic salmon in Norway are IPNV carriers (Havarstein *et al.*, 1990). In Norway, the incidence of clinical IPN in Atlantic salmon farms was 39% in 1991, and it had increased to 61% in 1995. The total loss from IPN in Atlantic salmon set to sea in Norwegian fish farms in 1995 was estimated to be 5%, giving economic losses about 60 million USD yearly (Christie, 1997). Smail *et al*., (1992) report an increased association of IPNV serotype Sp with cage sites and the failure of post-smolts to thrive in Scotland. Murray *et al*., (2003), used data from an official monitoring program to study the emergence of IPNV in Scottish fish farms from 1996 to 2001. Their results showed an 10% annual increase of IPNV in saltwater, a 2-3% annual increase in freshwater sites with a much faster annual increase of 6.5% in Shetlands freshwater sites. Murray *et al*., (2003) concluded that given IPNV prevalence of approximately 10% per year, effective control would have to be re-established very soon before it becomes ubiquitous in most areas, as for example in Shetland were the high IPNV levels stood out for both freshwater and seawater sites.

1.12 Control of IPN

1.12.1 Chemotherapy

No truly effective chemotherapeutics are available for the treatment of IPN (Stoskopf, 1993). Savan and Dobos (1980), used virazole to treat IPNV experimentally challenged rainbow trout, and found that treatment had only a slight positive effect. They suggested that repeated daily exposure of the fry to the virazole rather than the single exposure would have produced more promising results. However, Savan and Dobos (1980) stressed that in a hatchery situation, the cost involved in repeated exposure to virazole would be economically prohibitive, and as a result most hatchery owners would be reluctant to initiate any antiviral treatment until existence of the viral disease became apparent. Jashes *et al*., (1996) used a plaque evaluation assay to asses a group of compounds that had a broad spectrum antiviral activity for both single- and double- stranded RNA viruses. From their tests 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR) and 4-hydroxy-3-β-D-ribofuranosylpyrazole-5-carboxamide (pyrazofurin) both achieved inhibition of IPNV at concentrations that were 50-100 times lower than the concentration required to inhibit DNA synthesis in growing cells. Moya *et al*., (2000) developed these findings to test the *in vivo* antiviral effect of EICAR, by experimentally infecting coho salmon (*Onchorhynchus kisutch*) and rainbow trout fry with IPNV. The results showed that treatment with EICAR resulted in a reduction in the viral load of infected fish; however, this did not prevent them from being carriers. Therefore, it was concluded that treatment of EICAR could be effective for increasing salmon and trout production as it

reduces viral load and mortality, however as the transmission of IPNV is both horizontal and vertical, this treatment is ineffective for broodstock.

1.12.2 Vaccination

Vaccines have long proven their efficacy for the control of virus diseases, but in the aquaculture industry, they are in a relatively early phase of development. The high cost of new product development combined with the relatively small size of the industry and the low value of individual animals have largely contributed to this situation (Heppell and Davis, 2000). It is the opinion of Park and Jeong (1996), that once IPNV is established, it is very difficult to eradicate from infected fish, and the development of a safe, efficient and inexpensive vaccine against IPNV infection is greatly needed. According to Biering *et al.,* (2005) vaccines against infectious pancreatic necrosis (IPN) have been sold for many years in Norway and are now also available in Chile. Most of the research on these vaccines has been performed by pharmaceutical companies, and not much information is available as scientific publications. It has also been difficult to establish reproducible IPN challenge models suitable for vaccine testing and this probably explains the lack of scientific publications. There have also been a number of reasons offered as to why IPN vaccine development has proved so difficult. Wolf (1988) states that age of the fish that are susceptible to the disease and their lack of a developed immune system could be a factor in the lack of success in producing an effective vaccine against IPNV. It is the opinion of Noga (2000) that the large amount of serological variation among various strains and apparent lack of cross-protection has hindered development of a practical

vaccine. According to Dorson (1988), as a result of the epidemiological characteristics of IPN an vaccine should:

i) Protect the fish early in its life,

ii) Allow a rapid onset of protection,

iii) Be delivered easily i.e. orally, or best via immersion before fry start first feeding,

iv) Protect against a wide variety of antigenically different strains in view of a world wide use.

1.12.2.1 Recombinant vaccines

Nagy and Dobos (1987) produced monoclonal antibodies against IPNV and reported that all the neutralising monoclonal antibodies developed were VP2 specific, which led them to believe that VP2 contains the major neutralising epitopes of IPNV. Frost and Ness (1997) have described an IPN vaccine component developed from recombinant VP2 added to an existing multivariant injectable vaccine, against furunculosis, vibriosis and cold water vibriosis (Norvax protect-IPN; (NP-IPN)). Frost and Ness (1997) demonstrated that although the vaccine suppressed viral replication post-challenge, it did not produce any measurable humoral immune response. As trying to experimentally induce mortality or IPN pathology can be problematic (Sadasiv, 1995), due to the lack of a good challenge model the effect of this recombinant component can only be determined through the antibody response as demonstrated by Frost and Ness (1997). However, Biering (1997) used this same recombinant vaccine to immunize Atlantic halibut (*Hippoglossus hippoglossus*) prior to challenge with IPNV and was unable to show that it had a positive effect on the humoral immune system.

1.12.2.2 Live attenuated vaccines

According to Christie (1997), a live attenuated vaccine would be the most effective and inexpensive solution for IPN control, however finding a stable non-pathogenic strain of the virus has proven very difficult. Dorson *et al*., (1978) produced a non pathogenic variant from a wild IPN virus strain after several passages in rainbow trout gonad (RTG)-2 cells. This strain was used for infection of rainbow trout fry but was unable to provide any protection against an IPNV serotype Sp. The instability of attenuated vaccines has been highlighted as a potential drawback, (Wolf, 1988; Christie, 1997) with fears of live vaccines reverting to virulence (Dorson, 1988). These associated problems make the licensing of a live viral vaccines difficult (Bootland *et al*., 1990).

1.12.2.3 Inactivated vaccines

Dixon and Hill (1983) demonstrated that formalin and ß-propiolactone (BPL) could be used to inactivate IPNV for vaccine use. Injection is not considered to be a convenient method of administration in aquaculture, when large numbers of vaccine doses are required and when fish that require vaccination are small and thus difficult to handle. Bootland *et al.,* (1995) conducted an experiment to see if the immunization of adult fish with an inactivated IPNV vaccine would prevent the development of a carrier state, therefore resulting in IPNV free progeny. Bootland *et al.,* (1995) immunized adult brook trout broodstock 5 months prior to sexual maturity with an injection of inactivated IPNV in Freund's complete adjuvant. However, the inactivated IPNV vaccine failed to prevent the fish from becoming IPNV carriers and IPNV was detected in the faeces, blood components, organs and reproductive products of the immunized male and female fish. It

was therefore concluded that this vaccine preparation was unlikely to prevent vertical transmission.

1.12.2.4 DNA vaccines

Antiviral DNA vaccines carrying a gene for a major antigenic viral protein have received considerable attention as a new approach to vaccine development, especially when traditional vaccines have failed (Kim *et al*., 2000). DNA vaccines compared to traditional antigen vaccines have several practical and immunological advantages that make them very attractive for the aquaculture industry (Heppell and Davis, 2000). Kim *et al*., (2000) report that for fish viruses, DNA vaccines have been developed for IHNV and viral hemorrhagic septicaemia virus (VHSV), with laboratory trials indicating that these vaccines are more effective in protecting fish in challenge experiments than inactivated and subunit vaccines. Mikalsen *et al.,* (2004) reported that a DNA vaccine containing the whole large open reading frame (ORF) of segment A of the IPN virus provided a high level of protect in Atlantic salmon against a subsequent challenge of IPN.

1.12.3 Immunostimulants

Bricknell and Dalmo (2005) have stated that the theoretical benefit of immunostimulants is considerable, particularly given the current progress towards developing an efficacious IPNV vaccine. Immunostimulants have the potential to elevate the innate immune defence mechanisms of fish prior to the exposure to a pathogen, or improve survival following exposure to a specific pathogen. Sakai (1999) reviewed the use of fish immunostimulants; however, none of them have any documented effects towards IPN. Most of the studies that have been reported in literature have focused on the protection against bacterial pathogens

and/or non specific immune parameters such as phagocytic, complement or lysozyme activities, whilst studies on increased protection against viral infection are scarce. As a result, little is known about the antiviral effects of immunostimulants in fish (Salinas *et al.,* 2004). However, the findings of Damsgard *et al.,* (1998) may provide an obstacle to the delivery of immunostimulants through therapeutic diets to treat IPN. They reported that feed intake and growth were significantly lower in IPNV infected fish than uninfected fish, with some infected fish displaying a complete loss in appetite.

1.13 Husbandry

It is widely accepted that if IPNV has never been detected on a farm, every precaution should taken to prevent its introduction, including only stocking with inspected and certified IPNV free stocks (Blake *et al.,* 1995; Sadasiv, 1995). According to Alonso *et al.,* (1999), until vaccine technology improves, the only effective way to control virus infections in aquaculture is to prevent exposure to the virus. One example of this can be seen in Denmark, where IPN is considered to be endemic like most of Europe, except for a number of approved IPN-free rainbow trout broodstock farms. Most of these farms have been maintained as closed units since 1969 and have thus been able to uphold an IPN free status and have supplied IPN free material to the international market for decades (Ariel and Olesen, 2002). However according to Reno (1999), this type of vigilance is difficult to achieve, for economic or technical reasons. In practice, particularly in commercial operations where IPN cannot be avoided, economic loss is minimized by anticipating the extent of mortality and incubating proportionately more eggs (Wolf, 1988). It is the

opinion of Stoskopf (1993), that the incidence of acute IPN and consequent mortality can be reduced if factors that promote physiological stress are controlled. These included reducing population density, following optimal feeding protocols and maintaining proper hatchery hygiene.

1.14 Genetic resistance

Systematic breeding for increased innate resistance constitutes a potential strategy for control of infectious diseases in all segments of animal and plant production. According to Midtlyng *et al.*, (2002), because of the high reproduction rate and the opportunity to score families by use of challenge tests, fish have a much higher potential for improving resistance to infectious diseases through selective breeding than most other food producing animals. Some salmonid species are recognized as IPN resistant, e.g. coho salmon (Dorson, 1988).

1.15 Current techniques for the detection and identification of aquatic IPNV

Diagnostic procedures are important for IPN control. It is important that infectious agents are rapidly identified and differentiated, because rapid detection of an outbreak may help to prevent further spread of the disease (Espinoza and Kusnar, 2002). The diagnosis of IPN has historically been predicted on clinical signs of the disease, isolation and identification of the aetiological agent by cell culture methods and confirmation using

serological methods (Reno, 1999). However, according to (Ahne *et al*., 1989) clinical signs and histopathological changes associated with IPN are variable and cannot be used for presumptive or definitive diagnosis or to distinguish IPN from other fish viral diseases. In most cases, classification of these viruses as strains of IPNV are made on the basis of neutralization with antisera against reference strains of IPNV (Hill and Way, 1995). Immunofluorescence adsorption test (IFAT) is also used after conventional virus isolation on cell cultures, this method although time consuming, works very well in acute cases, but it is difficult to detect virus in subclinically or latently infected fish Barlic-Maganja *et al.,* (2002). According to Alonso *et al*., (1999), because of the occurrence of co-infections of IPNV with, for example IHNV, more sensitive detection methods for each virus are needed to avoid false negative results, as the growth of one virus may be inhibited by the other, and standard diagnostic assays might not reveal the second virus. Therefore, it is widely agreed that there is a need for a sensitive, rapid diagnostic technique (Lopez-Lastra *et al*., 1994; Hill and Way, 1995; Alonso *et al*., 1999; Taksdal *et al*., 2001; Espinoza and Kuznar, 2002). IPNV may be detected with molecular methods such as reverse-transcription PCR (RT-PCR). At present, the major disadvantage of molecular methods of virus detection is the cost, but the efficiency and rapidity compared with virus isolation and differentiation by cell culture methods, where 14-20 days are required for a negative diagnosis, offer a considerable advantage and offset the higher costs of molecular tests (Barlic-Maganja *et al.,* 2002). Blake *et al*., (1995) developed a RT-PCR assay which was found to be capable of routinely detecting aquatic birnaviruses directly in fish tissue samples at a level of accuracy and sensitivity comparable to those of virus isolation in cell culture. Taksdal *et al*., (2001), used RT-PCR to test Atlantic salmon for the presence of

IPNV in survivors of a viral bath challenge. Their results showed that RT-PCR detected a higher number of IPNV positive samples than standardised cell culture method detection. This was a significant finding as it was one of the first reported methods that was more sensitive than virus culture for the detection of IPNV. It is believed that methods that can detect IPNV in covertly infected fish may be beneficial in the surveillance and prevention of spread of the infection (Taksdal *et al*., 2001). According to Barlic-Maganja *et al.,* (2002) when RT-PCR positive results are obtained additional confirmation by culture based diagnostic methods is necessary and the latter will remain as the gold standard method for virus detection meantime. Therefore, it could be argued that molecular tests are inappropriate for final diagnosis of IPNV but are useful in conjunction with routine diagnostic procedures of virus isolation, especially when quick detection of viral agents could identify an outbreak and help to prevent further spread of disease. Therefore, according Einer-Jensen *et al*., (2002) cell culture assays are still considered to be the "Gold Standard".

1.16 Host defence mechanisms against IPNV

There is a belief that an increased knowledge about antiviral defence mechanisms of fish may contribute to the understanding of the development of virus diseases in aquaculture (Nygaard *et al*., 2000), and more specifically help to explain the susceptibility of Atlantic salmon to IPNV (Jensen and Robertsen, 2002).

1.16.1 Innate immunity

1.16.1.1 Cellular

According to Rønnesth *et al.,* (2006) the non-specific cellular immune activities are central in combating virus infections in fish. Cells with very similar properties to mammalian natural killer (NK) cells have been observed in fish (Ferguson, 1989). These natural cytotoxic cells (NCC) have cytotoxic effects on many tissue culture cells, especially when the latter are infected with IPN virus (Moody *et al*., 1985). According to (Ferguson, 1989) NCCs which are present in the blood, lymphoid tissues and mucosal sites, spontaneously kill cells via an apoptotic and necrotic mechanism. A variety of leucocyte types are involved in non-specific cellular defences of fish, and include monocytes/macrophages, granulocytes and non-specific cytotoxic cells (Moody *et al*., 1985). Macrophages play a significant role in non-specific resistance to virus infection, due in part to the non-permissiveness of these cells for the replication of many viruses (Nova *et al*., 1996). Another important phagocytic leucocyte is the neutrophilic granulocyte. In teleosts, three types of granulocytes, namely neutrophils, eosinophils and basophils, have been identified (Rønnesth *et al.,* 2006). Atlantic salmon parr and post smolts were challenged with IPNV and Rønnesth *et al.,* (2006) reported that there was a reduced level of neutrophils in the head kidney of infected parr and post-smolts than observed in non-infected fish. From their results, they suggested that neutrophils may take part in virus clearance or are affected by IPNV weeks post challenge. These results complemented other studies that report that IPNV effects the levels of neutrophils in IPNV infected Atlantic salmon (Pettersen *et al.,* 2003; Pettersen *et al.,* 2005). The cellular components of the non-specific immune system include phagocytic cells (neutrophils and

macrophages) and natural killer cells which interact with lymphocytes and other cells of the immune system through cytokines. Cytokines are polypeptides or glycoproteins which act as modulators in the immune system (Sakai, 1999). The production of T cell-derived cytokines able to influence phagocyte functions is a key aspect of cell-mediated responses (Mulero and Meseguer, 1998). In mammals, a diverse array of cytokines secreted by leucocytes is able to affect phagocyte activities (Mulero and Meseguer, 1998). There is evidence that fish, like mammals, have a network of signalling cytokines that control and coordinate the innate and acquired immune response (Magnadóttir, 2006). The cytokines that have been identified in fish are reviewed by Manning and Nakanashi (1996), which include Interleukin 1 (IL-1), Interleukin 2 (IL-2), Interleukin 3 (IL-3), Interleukin 4 (IL-4), Interleukin 6 (IL-6), Interferons (IFNs) and Macrophage activating factors (MAFs), Tumour Necrosis Factor (TNF), Transforming Growth Factor ß1 (TGF ß1), Chemotactic Factor (GF) and Macrophage Migration Inhibition Factor (MIF). IFNs are the most extensively studied cytokines regarding IPN, and their relevance will be discussed in the next section.

1.16.1.2 Humoral

The serum, mucus, and eggs of fish contain a variety of substances that non-specifically inhibit the growth of infectious microorganisms. They are specific in that they react with just one chemical group or configuration, but they have been called “non-specific” because they do not influence the growth of only one microorganism (Ahne and Negele, 1985). Another component of resistance to IPN is interferon, a broad ranging protective molecule generated by lymphocytes and other cells (Reno, 1999). Cells infected with a virus are stimulated to produce and secrete IFN, which in turn induces a complex pattern

of physiological changes, including the establishment of an antiviral state in as yet uninfected cells (Collet and Secombes, 2001). It is now appreciated that IFNs consist of two families, the type I or IFN-α/ß family that consists of many, structurally related members, and type II or IFN-γ, consisting of a single, unrelated protein (Levy *et al.,* 2001). Type I IFNs induce the production of antiviral proteins in various body cells whereas Type II IFNs, in addition to this, activates macrophages for enhanced killing of bacterial, fungal and viral pathogens (Robertsen, 1999). Viruses induce interferon gene expression and then the up-regulation of various downstream interferon responsive genes (Boudinot *et al.,* 2006; Bergan *et al.,* 2006). Some of these genes such as 2-5 A synthetase, RNA-dependant protein kinase, RNase I, and MxA, have antiviral activity (Boudinot *et al.,* 2006). It has been known for some time that interferon synthesis can be triggered in fish, either *in vivo* or in cell culture, following infection by pathogenic viruses (Hill, 1982). The limited number of functional studies that have been performed with the cloned fish IFNs show that they have the characteristic properties of type I IFNs (Robertsen 2005). Type I IFNs have been cloned from zebra fish (*Brachydanio rerio*); (Altmann *et al.,* 2003), Atlantic salmon (Robertsen *et al.,* 2003), channel catfish (*Ictalarus punctatus*); (Long *et al.,* 2004), whilst Mx genes have been detected and cloned in rainbow trout (Trobridge *et al*., 1997), Atlantic salmon (Robertsen *et al.,* 2003) and Japanese flounder (*Paralichthys olivaceus*); (Lee et al., 2000). Mx is one of several IFN-inducible proteins that have been shown to inhibit the replication of different types of viral infection. Mx proteins are members of a family of IFN-inducible genes expressed when cells undergo virus infection (Leong *et al*., 1998). The study of Mx-genes in cultured fish species is of importance not only as components of antiviral defense, but also as molecular markers for

type I IFN induction (Robersten *et al*., 1997). There is controversy as to whether IPNV induces IFN responses in fish cells (Collet *et al.,* 2007) which has led to suggestions of a complex virus/host interation (Lockhart *et al.,* 2006).

1.16.2 Adaptive immunity

1.16.2.1 Cellular

Cell-mediated killing is an important defence mechanism in the control of virus-infected cells (Nakanisihi *et al*., 2002; Somamoto *et al*., 2000). Virus-specific cell mediated cytotoxicity was demonstrated in fish for the first time by Somamoto *et al*., (2000) using clonal guibuna crucian carp (*Carassius auratus langsdorfiiand*) a syngenic cell line (CFS). Peripheral blood leucocytes, from crucian carp immunized with IPNV infected CFS cells lysed IPNV infected CFS cells (immunogen) more completely than CFS cells infected with different virus (non-immunogen). These results suggest that fish exhibit specific cytotoxicity against virus-infected cells, resembling the specific cytotoxicites of higher vertebrates. This appears to be the only study in the literature on specific cell-mediated immune responses to IPN in fish.

1.16.2.2 Humoral

According to Frost *et al.,* (1998) the importance of antibodies in a protective immune response against IPNV is unknown. Bootland *et al.,* (1991) reported that a strong antibody response was produced in 1 year old brook trout injected with IPNV, however this response failed to prevent a chronic infection which subsequently led to a carrier state within the survivors of the infection. Similarly Bootland *et al.,* (1995) attempted to immunize adult brook trout with inactivated IPNV, which induced a strong humoral

immune response with IPNV-neutralising antibodies. However, this failed to prevent the fish from becoming infected following challenge with IPNV. Biering (1997) performed an experiment in which Atlantic halibut were bath challenged with IPNV which subsequently led to a strong humoral immune response in the form of IPNV-specific serum antibodies. Whilst no mortality occurred as a result of virus exposure, no connection was observed between the high humoral immune response and virus elimination. Whilst the importance of IPNV-specific antibodies is unknown literature clearly indicates that they are a specific immune response.

1.17 Summary and Aims

IPN is the most serious viral disease affecting the UK salmon farming industry. Advancing current knowledge of the salmonid immune response to IPNV may highlight potential control measures against this major pathogen. The macrophage is an important component of the innate immune response. Although macrophages are recognised as a potential site of viral replication and persistence in IPNV-infected salmon, the virus-host relationship in this cell type is not well characterised. The salmonid IFN response to IPNV has been studied in cell lines such as RTG and chinhook salmon embryo (CHSE-214), however there is a distinct lack of knowledge of the interferon response to IPNV in salmon macrophages. Therefore, the overarching goal of this thesis is to characterise aspects of the antiviral response to IPNV in Atlantic salmon macrophages, and to investigate whether the antiviral mechanisms in these cells can be manipulated with immunostimulants so as to mitigate IPNV infection and thus potentially limit development of a carrier state.

This thesis comprises the following seven objectives:

- Development of methods for the isolation and *in vitro* culture of Atlantic salmon macrophages.
- Development of a procedure for the extraction of RNA from Atlantic salmon macrophages maintained *in vitro*.
- Establishment of real-time RT-PCR procedures for the quantitation of immune response gene expression in IPNV-infected Atlantic salmon macrophages and uninfected controls. This involves the development of assays to detect IFN and Mx, and also Elongation factor 1 (ELF-1). The latter is used as a "housekeeping" control gene in quantitative real-time RT-PCR.
- Establishment of a real-time RT-PCR procedure for the quantitation of IPNV in IPNV-infected Atlantic salmon macrophages and uninfected controls.
- To determine whether Atlantic salmon macrophages cultures maintained *in vitro* can be infected with IPNV.
- To characterise the effect of IPNV infection on expression of immune related genes in Atlantic salmon macrophages.
- To investigate whether the expression of immune related genes in IPNV-infected Atlantic salmon macrophages can be manipulated with immunostimulants. The following immunostimulants will be studied: Glucan, MAF, and LPS.

Chapter 2 - General Materials and Methods

2.1 Virology

2.1.1 Cell culture

A Shetland isolate of IPNV (IPNV 975/99); (Bowden *et al*, 2002) obtained from FRS Marine Laboratory, Aberdeen was grown in CHSE-214 cells. Cells were maintained in Eagles minimum essential medium (EMEM); (Gibco), without L-Glutamine, supplemented with 10% foetal calf serum (FCS); (Gibco), 2mM L-Glutamine (Gibco) and 1x non-essential amino acids (NEAA); (Sigma). The cells were cultured in closed 25 cm^2 plastic flasks (Nunc, Roskilde, Denmark) at 22˚C.

2.1.2 Virus culture

CHSE-214 cells were infected with IPNV by simultaneous inoculation. A $25cm^2$ culture flask of fully confluent CHSE-214 cells was split to a ratio of 1:3 and IPNV was inoculated at $1/10^{th}$ of the volume of the suspended cells. The cells and virus were incubated at 15°C. The cells were examined on a daily basis for the development of cytopathic effects (CPE), in comparison to uninfected control cells which were inoculated with Hanks' Balanced Salt Solution (HBSS) (Gibco). On observation of extensive CPE the supernatant was centrifuged at 1410 x g for 10 minutes at 5˚C (Eppendorf 5804R). The pellet was discarded and the virus-containing supernatant was aliquoted and stored at -70˚C.

2.1.3 Estimation of virus titre

The titre of IPNV preparations was performed by infectivity titration in CHSE-214 cells grown in 96 well plates (Nunc, Roskilde, Denmark). 90µl of EMEM supplemented with 10% FCS was added to each plate well together with 10µl of the IPNV preparation, which was then diluted in ten-fold dilutions across the plate. For each 96 well plate a fully confluent $25cm^2$ flask of CHSE-214 cells was harvested, and 100µl of CHSE-214 cells was added to each plate well. For a negative control, each plate contained two rows of CHSE-214 cells and EMEM. The plates were incubated for 7 days at 15ºC and inspected daily for the development of CPE. The titre of the virus expressed as TCID50 was calculated using the Spearman-Karber method (Hierholzer and Killington, 1996) as described below.

Log10 Median Dose = (X0 -(d/2) + d(∑ ri/ni)

X0 = log10 of the reciprocal of the lowest dilution at which all test inocula are positive.

D = log10 of the dilution factor (i.e. the difference between the log dilution intervals)

ni = number of test inocula used at each individual dilution (after discounting accidental losses)

ri = number of positive test inocula (out of ni).

∑(ri/ni) = ∑(P) = sum of the proportion of positive tests beginning at the lowest dilution showing 100% positive result.

Summation is started at dilution X0

2.2 Isolation and culture of macrophages

2.2.1 Fish

All the experiments conducted in this thesis used head kidneys derived from Atlantic salmon, (*Salmo salar* L.) of average weight 500g originating from the Marine Environmental Research Laboratory at Machrihanish.

2.2.2 Sampling

Prior to sampling the fish were killed by a lethal exposure to ethyl p-amino-benzoate (0.8 g/L) and ex-sanguinated by withdrawing blood from the caudal vein. The surface of the fish was sprayed with 70% ethanol (BDH) prior to dissection and the head kidney was aseptically removed and placed in 9ml of chilled Leibovitz medium (L-15); (Sigma) supplemented with 10% FCS, 50 U ml^{-1} penicillin (Gibco), 50 µg ml^{-1} streptomycin (Gibco), 2% polymyxin B sulphate (Sigma) (10 000 U ml^{-1}) and 2% gentamycin (Gibco) (50 mg ml^{-1}). The head kidneys was passed through a 100µm mesh with L-15 medium supplemented with 2% FCS, 1% penicillin/streptomycin (P/S) (Gibco) and 20 U ml^{-1} heparin (Sigma). All kidneys were processed within 24 hours of sampling.

2.2.3 Isolation of macrophages on 34-51% discontinous gradient

The head kidney cell suspension was split into three and was subsequently layered gently onto three 34-51% percoll (Sigma) gradients. The gradients were centrifuged at 540 x g

for 35 minutes at 4°C with no brake. The band of cells at the percoll interface was carefully collected so as not to disturb the 51% layer and washed with L-15 containing 0.1% FCS, 1% P/S and 20 U ml^{-1} heparin. The cells were centrifuged at 540 x g for 35 minutes at 4°C with no brake to wash off any percoll. After centrifugation the supernatant was removed and the pellet was resuspended in 1ml of L-15 medium.

2.2.4 Isolation of macrophages on 51% percoll

The head kidney cell suspension was split into three and was subsequently layered onto 51% percoll. The tubes were centrifuged at 540 x g for 35 minutes at 4°C with no brake. The band of cells at the medium-51% percoll interface was carefully collected so as not to disturb the 51% layer (as shown in Figure 1) and diluted in 10ml of L-15 medium. The cells were centrifuged at 540 x g for 35 minutes at 4°C with no brake to wash off any percoll. After centrifugation the supernatant was removed and the pellet was resuspended in 1ml of L-15 medium. The cells were counted using a haemocytometer and their viability was assessed by trypan blue exclusion. Cells were suspended to a concentration of 2 x 10^{-7} ml^{-1} in L-15 plus 0.1 % FCS.

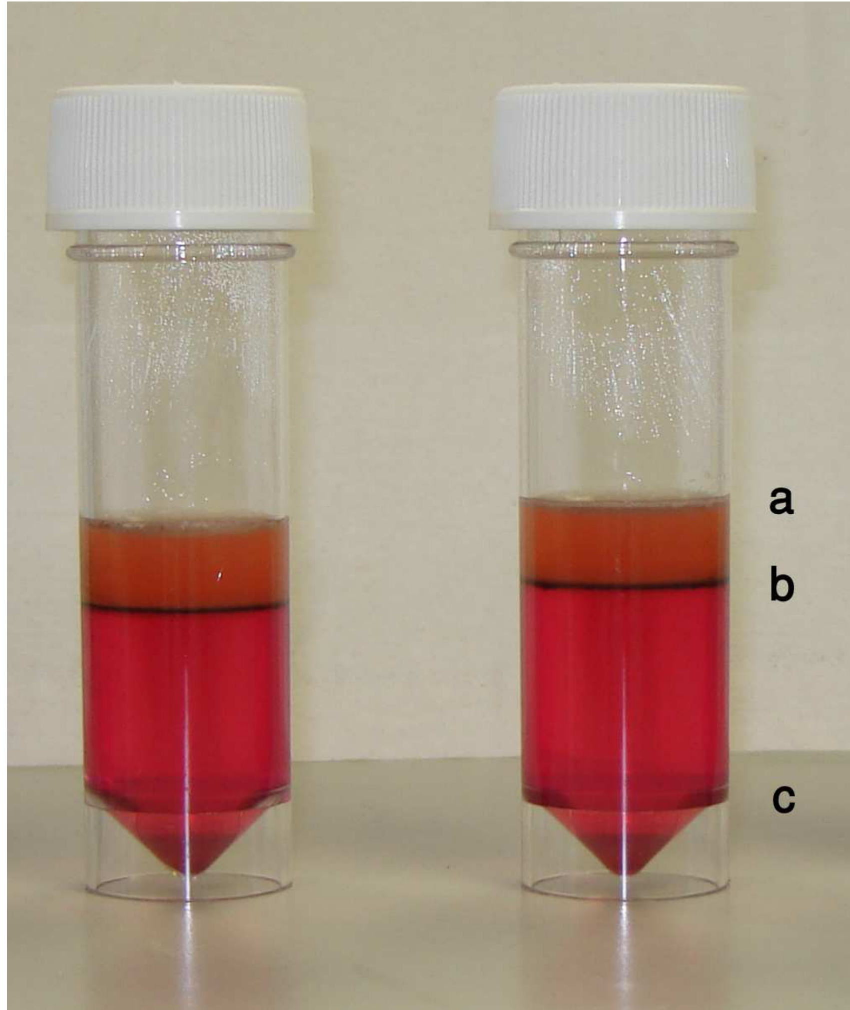


Figure 1: Isolation of macrophages on a 51% percoll gradient gave rise to 3 main layers. Layer 1; L-15 medium containing cell debris (a). Layer two; the L-15 medium-51% percoll interface containing macrophages (b). Layer three; 51% percoll with pelleted red blood cells (c).

2.2.5 Culture of macrophages

Aliquots of the cell suspension were added to culture plates, 100µl to each well of a 96-well culture plate whilst 400µl were added to each well of a 24-well culture plate, and the cells were left to attach at 15°C. After three hours the wells were washed three times with L-15 medium to remove any non-adherent cells. The adherent macrophage monolayers were maintained in L-15 medium supplemented with 50 units ml^{-1} penicillin, 50µg ml^{-1} streptomycin and 5% FCS at 15 °C. Over the course of the experiment, the medium was replaced every two days.

2.2.6 Estimation of macrophage cell numbers *in vitro*

Each day the number of viable macrophages was estimated. Following the removal of the culture medium from the well, lysis buffer (0.1 M citric acid, 1% Tween-20, 0.05% crystal violet). After 2 minutes the bean shaped nuclei were counted in a haemocytometer under an inverted microscope at 100x magnification.

2.2.7 IPNV infection of macrophages *in vitro*

Cell counts were performed in representitative wells to assess the numbers of viable macrophages 24 hours after initial washing of the macrophage monolayers to remove non-adherent cells. In the experimental wells the culture medium was removed and IPNV containing supernatant (section 2.1.2) diluted in L-15 medium was added at a multiplicity of infection (MOI) of 1 for 12 hours at 15°C. The virus titre of the IPNV containing

supernatant was 1.15×10^8 $TCID_{50}$ ml^{-1}. Control wells were inoculated in the same way with L-15 culture medium.

2.2.8 Extracellular IPNV titre

To determine if any IPNV had been released from the IPNV *in vitro* infected macrophages, 10µl of culture medium was removed from the macrophage culture wells on a daily basis and inoculated onto 24 well plates containing CHSE-214 cells. Had CPE been detected in these cultures over a 21 day period, 10ul of this culture medium would have been serially ten fold diluted and inoculated into 96 well plates containing CHSE-214 cells. These plates would then incubated for 7 days at 15°C and inspected daily for the development of CPE. The titre of the virus would then calculated using the Spearman-Karber method (Hierholzer and Killington, 1996) as described in section 2.1.3.

Chapter 3 - Development of a protocol for the *in vitro* culture of Atlantic salmon macrophages

3.1 Introduction

The macrophage is believed to be a key component in the salmon immune response to IPNV, and a possible site for persistent virus infection in this host (Johansen and Sommer, 1995). Consequently, *in vitro* cultures of salmon macrophages represent a useful model to study aspects of the relationship between IPNV and its host. This model system would also permit experimental investigation of the effects of immunostimulants on the immune response of the macrophage to IPNV. The objectives of this chapter are to investigate the effect of isolation method and culture conditions on macrophage preparations used for *in vitro* culture.

3.2 Materials and Methods

3.2.1 Isolation of macrophages on 34 - 51% discontinuous percoll gradient or 51% percoll.

Head kidneys were obtained from the fish as outlined in section 2.2.2. Macrophages were isolated using either 34 – 51% percoll gradients (section 2.2.3) or using 51% percoll (section 2.2.4). The cells were seeded into either 96 or 24 well plates at concentrations of 2 x 10^{-7} ml^{-1} and maintained in culture for 9 days post isolation at 15°C as described in section 2.2.5.

3.2.2 Statistical analysis of the cells produced by the 34-51% percoll gradient and those on 51% percoll

Cell counts were performed regarding the number of cells produced from 6 different 34-51% and 51% percoll isolation preparations, using a haemocytometer and their viability was assessed by trypan blue exclusion. Triplicate counts were recorded for each preparation. Comparisons between the numbers of cells produced by the two isolation methods were analysed by a Mann and Whitney test, using the Minitab software package. Differences were considered statistically significant when probability (P) values < 0.05 were obtained.

3.2.3 Staining of adherent cells obtained from 34-51% and 51% percoll gradients

Microscope slides were cleaned in 100% ethanol. Two circles were drawn on each slide using a PAP pen (AGAR scientific). 100µl of cell suspension from the 34-51% and 51% percoll gradients were placed in each circle. The cells were left to attach for three hours at 15ºC. The non adherent cells were removed by washing the slides the slides three times with L-15 medium and gently tapped dry. The slides were dipped in 70% ethanol to fix the cells and then stained with a Quick stain kit (Raymond Lamb Ltd) following the manufacturer's instructions. The cells were then mounted using pertex and viewed under oil immersion at x 100 magnification.

3.2.4 *In vitro* virus infection in isolated macrophages

Macrophages maintained in 96 and 24 well plates were infected, in triplicate, with IPNV at a multiplicity of infection (MOI) of 1 (section 2.2.7) and incubated overnight at 15°C. The next day, the cells were washed three times with L-15 medium and then maintained in culture for nine days at 15°C as described in section 2.2.5. Control wells of macrophages were inoculated in the same way with L-15 culture medium.

3.2.5 Extraction of RNA from *in vitro* macrophage monolayer

Each day over a 9-day period, the supernatants were removed and total RNA was isolated from the macrophage monolayer using TRIzol reagent (Gibco) following the manufacturer's protocol. Triplicate wells were sampled for each time point. The resulting RNA was resuspended in 10µl of RNase/DNase free water, left to sit on ice for 2 hours to allow satisfactory resuspension of the pellet and the concentration was estimated from optical density (OD) measurements performed on a NanoDrop spectrophotometer (NanoDrop Technologies). In addition, total RNA was isolated from the macrophage monolayers in 24 well plates using a modified version of the TRIzol procedure incorporating the use of a commercial co-precipiatant, Glycoblue (Ambion) and also an overnight precipitation step at -20ºC. The modified procedure is as follows. Each day over a nine day period the supernatants were discarded and the macrophages were lysed by adding 800µl of TRIzol reagent to each well for 5 minutes at 15-30ºC and the resulting cell lysate was passed through a pipette several times and passed into a sterile

diethylpyrocarbonate (DEPC)-treated 1.5ml tube. To each tube, 160µl of ice cold chloroform was added and the tubes were shaken vigorously by hand for 15 seconds. The tubes were incubated at 15-30ºC for 2-3 minutes before centrifugation at 12,000 x g for 15 minutes at 4ºC. The clear aqueous phase was transferred to a new sterile DEPC-treated 1.5ml tube, to which a co-precipitant, GlycoBlue (Ambion) was added at a concentration of 150µg/ml prior to the addition of 400µl ice cold isopropyl alcohol. The tubes were gently inverted 7 times and the samples were allowed to precipitate overnight at -20ºC. The following day samples were centrifuged at 12,000 x g for 15 minutes at 4ºC to pellet the RNA. The supernatant was discarded and the pellet was washed with 200µl ice cold 70% ethanol and centrifuged at 7,500 x g for 10 minutes at 4ºC. The supernatant was removed and the pellet was allowed to dry. Once dried the pellet was resuspended in 10µl RNase/DNase free water and left on ice for 2 hours to ensure adequate resolubilization before the concentration of extracted RNA was assessed. The concentration was estimated from Optical density measurements performed on a Nanodrop spectrophotometer.

3.2.6 Viability of *in vitro* macrophage cultures

Prior to harvesting, the number of viable adherent macrophages in representitative wells was determined as outlined in section 2.2.6.

3.3 Results

3.3.1 *In vitro* culture of macrophages and extraction of RNA from cells grown in a 96 well plate

Macrophages were not able to survive past 4 days post infection as reflected by the cell counts in Table 1. It was found that the macrophage monolayer was either significantly or almost totally destroyed after day 3 of culture, as reflected in the steep fall in cell numbers (Figure 2). This coincided with the 3rd wash, as the cells were first washed after the initial incubation, washed again after being inoculated with virus, and then again after a second day in the culture medium as recommended by Secombes (1990). The pattern of damage to the monolayer was evidently linked to the washing of the cells as when the wells were viewed under the microscope after each wash the macrophage monolayer was greatly reduced compared to before washing. There was also a distinctive pattern in which the macrophages detached and this corresponded to where the washing medium impacted on the culture surface of the wells. To perform the experiments proposed for this thesis the monolayer would be required to withstand many washes to accommodate the various treatments required to perform this project. Together with the maintenance wash required every 48 hours, this would lead to considerable physical stress being imposed upon the macrophage monolayer during the duration of experiments. After many attempts to maintain an adequate macrophage culture in a 96 well plate it was decided that an alternative culture vessel would need to be used. It was also found that sufficient quantities of RNA for RT-PCR analyses could not be obtained from damaged macrophage cultures. Throughout the nine days, it was not possible to detect the presence

of any RNA after conducting extraction from the culture wells. As a result no RT-PCR amplification from this procedure was performed.

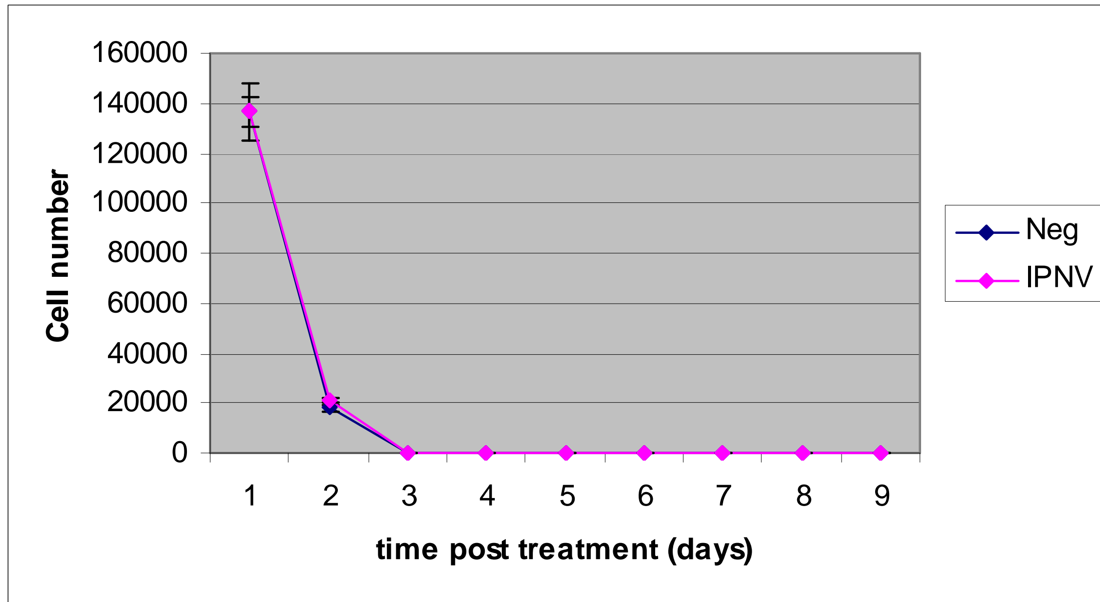


Figure 2: Number of viable adherent head kidney macrophages maintained *in vitro* over time in a 96 well plate. Cells were derived from Atlantic salmon head kidney and isolated on 34-51% percoll gradients. The cells were seeded in wells at a concentration of 2 x 10^{-7} ml^{-1}, then washed after 3 hours to remove non-adherent cells. Cultures were incubated overnight at 15°C, before being infected with IPNV at an MOI of 1, 24 hours after initial seeding. Negative control macrophages were inoculated with L-15 medium. Counts of viable macrophages were taken for 9 days after virus inoculation. Data represent mean counts (N=3) ± SD.

Table 1: Data collected from cell counts of viable adherent head kidney macrophages maintained *in vitro* over time in a 96 well plate after IPNV inoculation. Data represent mean counts (N=3) ± SD.

	Neg		IPNV	
Day	Mean	Stnd dev	Mean	Stnd dev
1	136666.7	11547.01	136666.7	5773.503
2	18000	1000	21000	1000
3	103.3333	90.73772	86.66667	75.71878
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0
9	0	0	0	0

3.3.2 Comparison of cells isolated from 34-51% percoll gradient and 51% percoll

In light of the problems encountered with maintaining macrophages in 96 well plates it was proposed that it might be necessary to culture the cells in larger wells. The use of larger volume culture wells might not only reduce the damage caused by washing, but would also yield more cells due to their larger surface area. This would facilitate the recovery of larger amounts of RNA from cultured macrophages. To support this larger culture well format more macrophages would therefore be necessary. As 34-51% percoll gradients are very labour demanding it was proposed that simply isolating macrophages on 51% percoll may be a better suited technique to meet the increased demand for cells. However, before this move could be made a comparison was made between the cells isolated from 34-51% percoll gradients and those from 51% percoll. On visual examination of the cells stained on the slides, it was confirmed that macrophages were

present after the three hour wash in both cell suspension preparations obtained using 34-51% percoll gradients (Figure 4) and 51% percoll (Figure 5). Further verification was provided through statistical analysis, performed on the cell counts immediately after isolation (section 3.2.2), which showed that there was no significant difference between the number of macrophages present in the cell suspension produced by the two methods ($P = 0.4290$). Therefore, it was concluded that the 51% percoll isolation method was a suitable replacement for the 34-51% percoll gradient isolation method.

```
34/51 Percoll  gradient  N =  18     Median =      5.9000
51 Percoll               N =  18     Median =      5.9000
Point estimate for ETA1-ETA2 is      0.0000
95.2 Percent CI for ETA1-ETA2 is (-0.0000,0.1000)
W = 358.5
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.4290
The test is significant at 0.4037 (adjusted for ties)

Cannot reject at alpha = 0.05
```

Figure 3: Mann and Whitney Statistical test preformed on macrophage counts obtained from cell suspensions derived from Atlantic salmon head kidneys. Comparisons were made concerning the numbers of macrophages obtained from 34-51% percoll gradients and those on 51% percoll ($N=18$). Differences were considered statistically significant when $P < 0.05$.

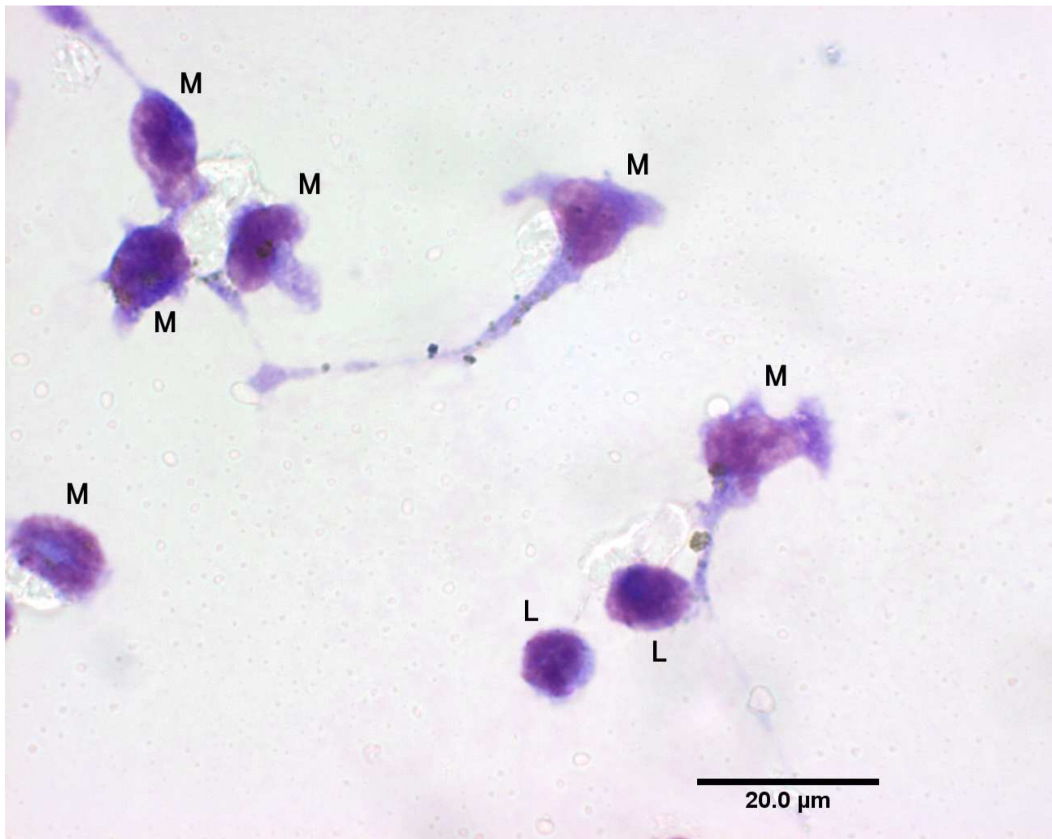


Figure 4: Adherent cell populations obtained following isolation on 34-51% percoll gradients. Aliquots of cell suspension at the 34-51% percoll interface were allowed to adhere to glass slides for 3 hours. Unattached cells were removed by washing and the remaining adhered cells were stained. Cells were identified as being either macrophages (M) or Lymphocytes (L). Photographs were taken using an Olympus BX51 inverted microscope under oil immersion at x 100 magnification.

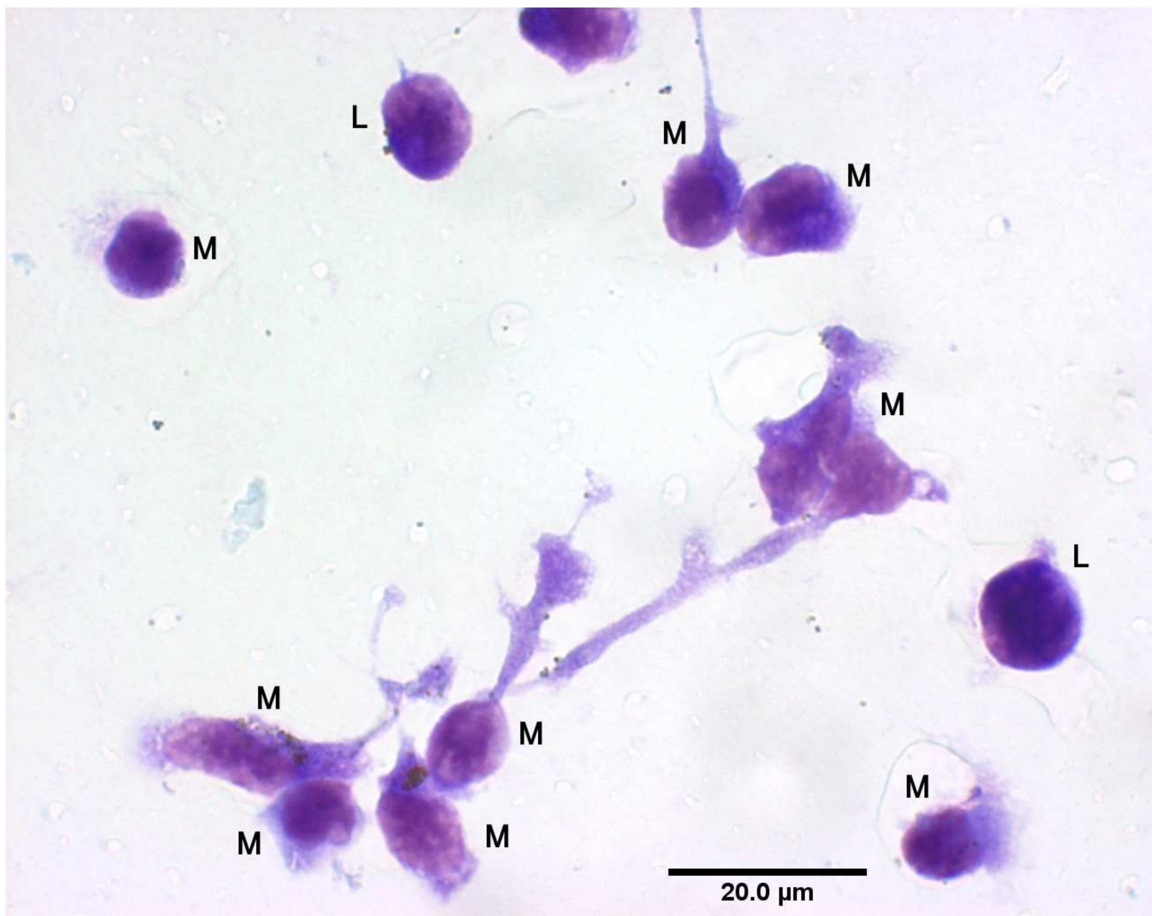


Figure 5: Adherent cell populations obtained following isolation on 51% percoll. Aliquots of cell suspension at the 51%-medium interface were allowed to adhere to glass slides for 3 hours. Unattached cells were removed by washing and the remaining adhered cells were stained. Cells were identified as being either macrophages (M), or Lymphocytes (L). Photographs were taken using an Olympus BX51 inverted microscope under oil immersion at x 100 magnification.

3.3.3 *In vitro* culture of macrophages and extraction of RNA from cell monolayers grown in 24 well plates

Through the use of 24 well culture plates it was possible to maintain macrophages in culture for nine days (Figure 6). The larger wells could be washed without total destruction of the macrophage monolayer as for the first time it was possible to obtain cell counts for each day of the experiment Table 2. The 24 well plate format meant that a multi-channel pipette could not be used for washing and it was necessary to use a 1ml pipette for this purpose. Despite the fact that this increased the time required to wash the plates, in essence, each well could be washed individually, and thus more care and attention could be allocated to each individual well. The effect of the washing was confined to a small area around the outer side of the well, thus leaving a monolayer of attached macrophages on the remainder of the culture surface of the well. RNA was extracted from the macrophages on a daily basis using TRIzol following the manufacturer's protocol. However, the extractions failed to reliably provide sufficient RNA to permit analysis of gene expression by real time RT-PCR (Figure 7). Previously, the 96 well plates was a major contributing factor to inability to obtain RNA yields. However, with using a culture system such as the 24 well plate which is able to maintain cells over the required experimental period, contributory factors could also include the loss of RNA during the extraction method, arising from a small amount of starting material. In this case, it is easy to lose pelleted RNA during extraction due to lack of visibility. To combat this it was decided to include a co-precipitant into the extraction process to increase the efficiency of RNA precipitation and to increase the visibility of the RNA pellet. In addition, an overnight precipitation step was included. By modifying

the TRIzol extraction method and incorporating the use of a co-precipitant it was possible to consistently extract a high yield RNA from macrophages (Figure 8). Extractions yielded on ranged between 115ng/µl to 400ng/µl of total RNA per well of a 24 well plate throughout the duration of the experimental time course. This corresponded to a total yield between 1150ng and 4000ng of total RNA per well.

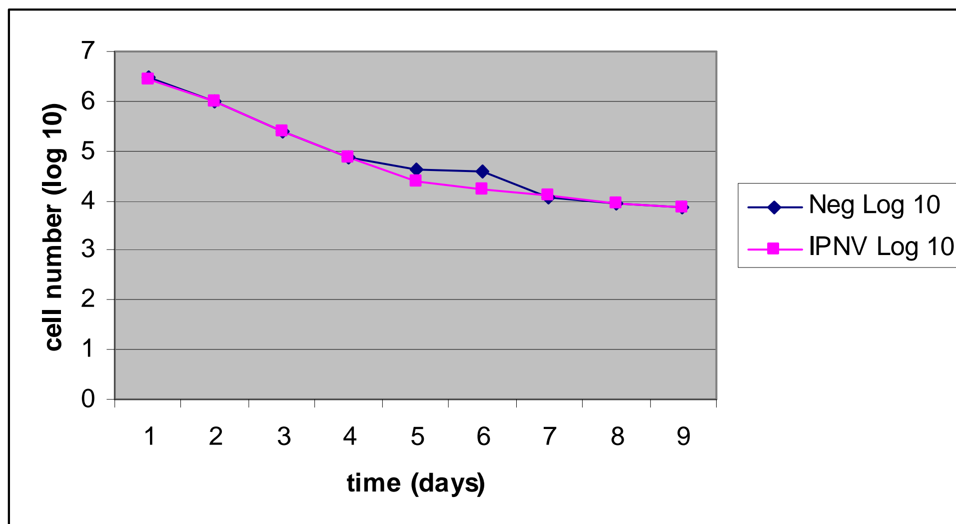


Figure 6: Number of viable adherent head kidney macrophages maintained *in vitro* over time in a 24 well plate. Cells were derived from Atlantic salmon head kidney and isolated on 51% percoll. The cells were seeded in wells at a concentration of 2 x 10^{-7} ml^{-1}, then washed after 3 hours to remove non-adherent cells. Cultures were incubated overnight at 15°C, before being infected with IPNV at an MOI of 1, 24 hours after initial seeding. Negative control macrophages were inoculated with L-15 medium. Counts of viable macrophages were taken for 9 days after virus inoculation. Data represent mean counts (log 10); (N=3) ± SD.

Table 2: Data collected from cell counts of viable adherent head kidney macrophages maintained *in vitro* over time in a 24 well plate after IPNV inoculation. Data represent mean counts (N=3) ± SD.

	Neg		IPNV	
Day	Mean	Stnd dev	Mean	Stnd dev
1	2866667	152752.5	2833333.333	115470.0538
2	966666.7	15275.25	963333.3333	5773.502692
3	236666.7	15275.25	236666.6667	11547.00538
4	70666.67	1527.525	71000	1732.050808
5	43333.33	577.3503	23666.66667	1154.700538
6	37666.67	1154.701	17333.33333	1154.700538
7	11666.67	1527.525	12333.33333	1527.525232
8	8600	200	8633.333333	115.4700538
9	7366.667	152.7525	7300	173.2050808

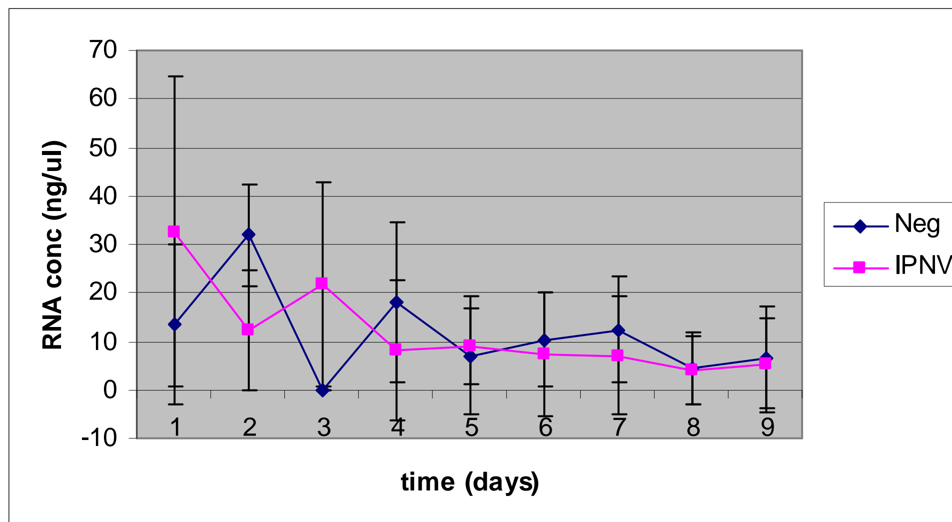


Figure 7: Yield of RNA extracted from adherent head kidney macrophages maintained *in vitro* over time in a 24 well plate. Cells were derived from Atlantic salmon head kidney and isolated on 51% percoll. Prior to extraction, the macrophages had been infected with IPNV at an MOI of 1, 24 hours after initial seeding. Negative control macrophages were inoculated with L-15 medium. RNA was extracted from macrophage monolayers for 9 days after virus inoculation; extractions followed the TRIzol manufacturer's protocol. Data represent mean concentration (N=3) ± SD.

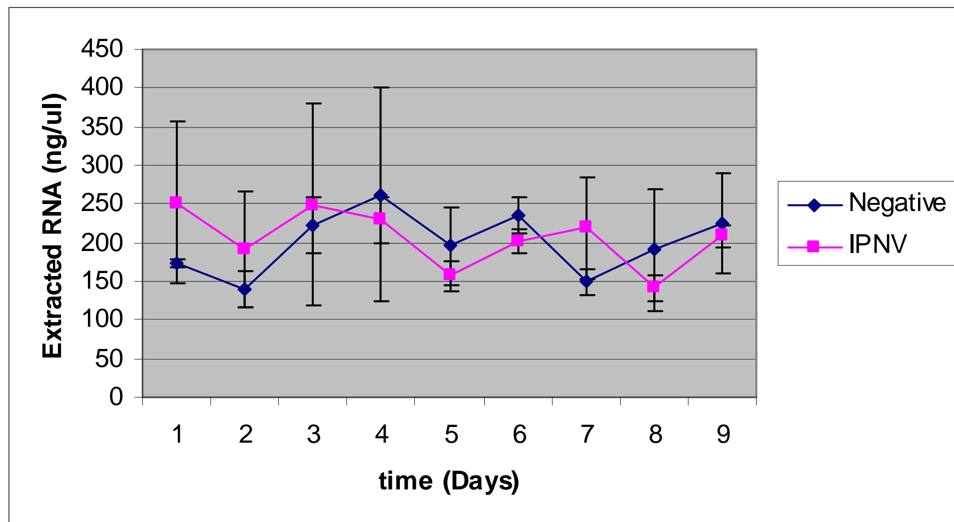


Figure 8: Yield of RNA extracted from adherent head kidney macrophages maintained *in vitro* over time in a 24 well plate. Cells were derived from Atlantic salmon head kidney and isolated on 51% percoll. Prior to extraction, the macrophages had been infected with IPNV at an MOI of 1, 24 hours after initial seeding. Negative control macrophages were inoculated with L-15 medium. RNA was extracted from macrophage monolayers for 9 days after virus infection. Extractions were performed using a modified version of the TRIzol manufacturer's protocol, which included the an overnight precipitation step at -20°C and the use of a co-precipitant. Data represent mean concentration (N=3) ± SD.

3.4 Discussion

3.4.1 Macrophage isolation conditions

Whilst the results of this chapter show that it was eventually possible to maintain an adequate culture of macrophages for 9 days, the use of a 24 well culture plates was not without problems. Firstly, increased numbers of macrophages were required for seeding wells. Originally, only 100µl of macrophage suspension was needed per well, however 400µl of macrophage suspension were required to seed the larger sized wells. Obtaining enough macrophages to satisfy the number of replicates needed to conduct an experiment would involve the use of significantly more percoll gradients, which are costly to produce and labour intensive to process. The most obvious solution to this problem was to implement the use of an alternative isolation technique that would lend itself to isolating large amounts of macrophages from salmon head kidney. As 51% percoll gradients are easier to prepare and faster to process than 34-51% gradients, their use permits macrophage isolation in reduced time, which is beneficial when several macrophage isolations are required to set up a single experiment. It was also feared that the large numbers of gradients required would increase the delay between removal of macrophages from transport medium and final transfer to culture plates. It was believed that this would have a detrimental effect on the physical condition and health of the macrophages, which could subsequently affect their ability to survive in culture. The use of 51% gradients allowed a faster processing time as it took less time to make these gradients and it was also quicker to load the kidney homogenate as the 51% percoll gradients are more stable than 34-51% gradients. This permitted processing of a greater number of kidneys which

was necessary to perform the number of replicates required for the experiments planned for this thesis. Originally, there was concern that using 51% percoll gradients would be less efficacious than the use of 34-51% gradient. It was anticipated that that the 51% gradient technique would almost certainly produce a less pure macrophage preparation compared to the 34-51% gradient technique, however it was hoped this would not hinder the macrophages present in the end cell suspension from adhering. As long as macrophages were allowed to adhere to the surface of the culture well there was confidence that the washing would make use of the attaching properties of the macrophages to remove the other cell types that may have been present in the wells. As Figure 4 and Figure 5 show, as a result of the wash after 3 hours, it was possible to show the appearance of macrophages from either isolation protocol. As there was no significant difference between the 34-51% percoll gradients and the 51% in terms of the number of macrophages present in the isolated cell suspension, the 51% percoll gradient isolation technique was considered to be a suitable procedure for isolating macrophages for the *in vitro* studies described in this thesis. Finally, a commonly overlooked factor in choosing a suitable experimental technique is financial cost, a factor which is of crucial importance for small scale research projects with limited financial support. The use of 51% gradients to obtain macrophages permitted a reduction in the amount of percoll used. Percoll is an expensive chemical and therefore lowering its consumption reduced the overall cost of the isolation method. Although 34-51% percoll gradients are the traditional way to isolate macrophages (Secombes, 1990; Nova *et al.,* 1996; Taffalla and Nova, 2000; Munro *et al.,* 2004), 51% gradients have previously been used to successfully isolate macrophages for

in vitro studies (Jorgensen and Robertsen, 1995; Sangrador-Vegas *et al.,* 2000; Munzo *et al.,* 1999) and 54% gradients (Rise *et al.,* 2004).

3.4.2 *In vitro* culture of macrophages and extraction of RNA

Initially growth of macrophages on 96 well plates was attempted, as this would require the use of fewer experimental animals, an important consideration given the ongoing debate on the use of animals in scientific research. However, as a result of poor survival of isolated macrophages, it was necessary to use 24 well plates. By using a 24 well culture plate, it was possible to maintain the macrophages in culture for at least 9 days post infection with IPNV. A similar pattern of viable cells in the IPNV infected and uninfected macrophages over time as shown in Figure 6 would confirm that the decrease in cell number over time in a direct result of washing and natural cell death/detachment in the monolayer and not as it could have been suggested due to the actions of the inoculated virus. Johansen and Sommer 1995 and Collet *et al.*, 2007, also report that IPNV infected macrophages can be maintained in vitro for at least 7 and 9 days post infection, respectively, without the development of CPE in the cells. It is probable that this would have been necessary even if better macrophage survival occurred in the 96 well plate format. This is because even with 24 well plates, in some cases it was difficult to obtain sufficient cells to perform RNA isolation and subsequent first strand amplification. This is especially relevant for studies investigating low copy transcripts that represent a small proportion of the total RNA yield. Whilst Table 2 shows that the number of macrophages declines throughout time, the remaining cell numbers were proven sufficient to obtain an adequate amount of RNA for subsequent amplification

Figure 8. However, this was only possible after the TRIzol extraction technique was developed so that it was sensitive enough to yield sufficient RNA for subsequent real-time RT-PCR analysis. It was necessary to adapt the manufacturer's protocol to increase the efficiency of the precipitation step. The inclusion of a co-precipitant into the extraction method proved to be a crucial part of the extraction procedure as before its addition it was not possible to routinely visualise RNA pellets. Sørensen *et al.*, (1997) reported that in an *in vitro* culture of Atlantic cod (*Gadus morhua* L) macrophage 95% of the cells had detached and died by 7 days, however they were able to maintain the culture for up to 3 weeks when the medium was carefully changed every third day. Similarly Johansen and Sommer (1995) observed that after 3 days in culture, about 30-40% of the adherent Atlantic salmon macrophage *in vitro* culture was still viable, however only 10% maintained adherence at day 7. The effect of the decline in the numbers of macrophages during the course of the experiment will not influence the results of gene expression, as the method of quantitation selected for this thesis is relative expression. Therefore, the expression of each target gene of interest will be compared to the expression of an internal control. The long term viability of the macrophage cultures was further reflected through the uniform ELF-1 Ct values across day 1-day 9 time points (section 5.3.1). This would indicate that the *in vitro* culture conditions do not influence the expression of the internal control gene, therefore demonstrating that the cell is stable in culture.

3.5 Conclusion

Although the need use a culture plate with a larger surface area was necessary to achieve adequate cell survival and sub-sequential RNA yield for real time RT-PCR amplification, the demand for increased cell numbers to successfully conduct an *in vitro* experiment was counterbalanced by identifying a optimal cell isolation and RNA extraction technique. According to Braun-Nesje *et al.,* (1981) the ability to separate, identify and maintain cells under *in vitro* culture conditions can facilitate the study of immunity in Atlantic salmon. In the present study, a reliable isolation and culture method for macrophages and a reproducible RNA extraction method have been developed that can be used to study the interaction between macrophages and IPNV. In conjunction with real-time RT-PCR assays for specific components of the innate immune system, macrophage culture *in vitro* may help to advance the understanding of IPN and thus contribute to the control of this disease.

Chapter 4 - Optimization of qRT-PCR assays for the quantitation of the expression of immune response genes in Atlantic salmon macrophages

4.1 Introduction

PCR is a powerful tool for the amplification of small amounts of DNA or RNA for various molecular analyses (Wang *et al.,* 1989). The introduction of real-time PCR has revolutionised quantification with this procedure, but requires careful assay design and reaction optimisation to maximize sensitivity (Peters *et al.,* 2004). According to Bustin and Nolan (2004), it is vital to consider each stage of the experimental protocol, starting with the laboratory setup and proceeding through sample acquisition, template preparation, reverse transcription, and finally amplification. Only if every one of these stages is properly validated is it possible to obtain reliable quantitative data. The aims of this chapter are;

a) To establish real time RT-PCR (q RT-PCR) assays to detect IFN, Mx, IPNV and ELF-1.

b) To identify a suitable quantitative model to investigate expression of IFN, Mx relative to ELF-1 in macrophages maintained *in vitro* throughout the course of an experiment.

4.2 Materials and Methods

4.2.1 Isolation of macrophages from head kidney

Head kidneys were obtained from Atlantic salmon (section 2.2.2) and macrophages were isolated on 51% percoll as described in 2.2.4. Macrophages were seeded into 24 well plates at concentrations of 2 x 10^{-7} ml^{-1} and maintained at 15°C as described in section 2.2.5.

4.2.2 IPNV infection of Atlantic salmon macrophages *in vitro*

After 24 hours incubation at 15°C, macrophage monolayers were processed in triplicate using one of the following two treatments.

a) IPNV-infected group. Macrophages were infected with IPNV at a multiplicity of infection (MOI) of 1 (section 2.2.7).

b) Poly-I:C-stimulated group. Macrophages were incubated with L-15 medium supplemented with 5% FCS and 50 units ml^{-1} penicillin, 50µg ml^{-1} streptomycin containing polyinosinic-polycytidylic acid (Poly I:C) (Sigma); (25 µg ml^{-1}).

After 24 hours incubation at 15°C, the monolayers were washed three times with L-15 medium and the RNA was extracted using the modified TRIzol protocol as described in section 3.2.5. It was necessary to reduce the post-infection sampling time from 24 hours to 12 hours to assess the amplification efficiency of the IFN primer/probe set because of the results of chapter 6 with respect to the time course of IFN expression.

4.2.3 Two-step RT-PCR

4.2.3.1 First strand cDNA synthesis

RNA from IPNV-infected or Poly I:C stimulated Atlantic salmon head kidney macrophages was selected to optimize the reverse transcription step, as it was considered that the RNA originating from these cells would contain all of the mRNA target sequences of interest in this study. The optimization was performed using TaqMan® Reverse Transcription reagents (Applied Biosystems) following the manufacturer's recommendations. This involved varying the reaction temperatures in the range of 42°C to 52°C, and the reaction times between 20 to 60 minutes. Due to cost restrictions the reaction temperatures studied were 42°C, 46.4°C and 52°C, whilst the reaction times were increased in 10-minute increments. cDNA synthesis was performed using TaqMan® Reverse Transcription reagents. The reaction mixtures for the RT reactions followed the guidelines in the TaqMan® Reverse Transcription reagents protocol; for each 10µl reaction; 1x 10x TaqMan RT buffer, 5.5 mM 25mM Magnesium Chloride, 500µM of each dNTP in a deoxyNTP mixture (deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate) 2.5µM random hexamers, 0.4 U/µl RNase inhibitor and MultiScribe Reverse Transcriptase (50 U/µl) 1.25 U/µl. The RT mix containing random hexamers was heated to 95°C for 10 minutes to denature the secondary structure of the IPNV template. After heating, samples were held on ice for 2 minutes before the second step amplification on the Rotorgene real-time thermal cycler (section 4.2.3.2). Again, as a result of financial restraints reverse transcription and amplification of only one of the target genes of interest, Mx and the housekeeping gene ELF-1 were optimized. Results were used

to define common optimal amplification conditions which would then be used for all amplifications.

4.2.3.2 IFN, Mx, IPNV and ELF-1 q RT-PCR

Primer and probe sequences for IFN, Mx, IPNV and ELF-1 were kindly provided by B. Collet, FRS Marine Laboratory, Aberdeen (Table 3). All primers and probes were designed and synthesized by Applied Biosystems. The probes were` 5' labelled with the fluorescent reporter molecule FAM (carboxyfluorescein), whilst the 3’ termini were modified with an minor groove binding (MGB) moiety. Real-time quantitative PCR was preformed on a Corbett Research Rotor-Gene. Each 20µl reaction contained: 1x TaqMan® Universal PCR mastermix (Applied Biosystems), 0.9mM forward and reverse primers, 0.25mM MGB probe. Cycling conditions were as follows: 10 min at 95°C, followed by 40 cycles of 92°C for 15 seconds and 60°C for 1 minute. All of the probes were designed to flank an RNA splice site in order prevent amplification of genomic DNA.

Table 3: Primer and probe nucleotide sequences used in real-time qRT-PCR

Gene	Forward primer	Reverse primer	MGB probe
IPNV	5’GCCAAGATGACC CAGTCCAT 3’	5’TGACAGCTTGAC CCTGGTGAT 3’	6-FAM-CCGACCGAG AACAT-MGB
Mx	5’GATGCTGCAC CTCAAGTCCTATTA 3’	5’CGGATCACCA TGGGAATCTGA 3’	6-FAM-CAGGATATCC AGTCAACGTT-MGB
Type 1 IFN	5’ACTGAAACGCT ACTTCAAGAAGTTGA 3’	5’AGGAAAGAGAC AAAACGTCATCTGC 3’	6-FAM-CTGTGCACT GTAGTTCATTT-MGB
ELF-1	5’CCCCTCCAG GACGTTTACAAA 3’	5’CACACGGCC CACAGGTACA 3’	6-FAM-ATCGGTGGTA TTGGAAC-MGB

4.3 Reverse transcription of total RNA extracted from macrophages incorporating the optimised cycling conditions.

All samples were diluted to a concentration of 100ng/µl in RNase/DNase free water. Amplifications were performed using TaqMan® Reverse Transcription reagents. The reaction mixtures for the RT reactions followed the guidelines recommended in the TaqMan® Reverse Transcription reagents protocol as described in 4.2.3.1. Prior to cycling, the amplification mix was heated to 95°C for 10 minutes and then placed back on ice. The RT conditions were as recommended in the TaqMan® Reverse Transcription reagents and incorporated optimised reaction duration and temperatures identified from the first strand synthesis optimization experiment (section 4.5.1). These were 10 minutes at 25°C, 60 minutes at 46.4°C, 5 minutes at 95°C.

4.4 Standard curve production

Standard curves of real-time RT-PCR experiments were produced by amplifying a ten fold dilution series of target in RNase/DNase free water. A master mix was prepared and distributed into 0.2ml tubes prior to the addition of template. Amplifications were performed in triplicate under optimised conditions. On completion of the run, a standard curve was produced using the Rotorgene software Version 6, which was also used to calculate amplification efficiency.

4.5 Results

4.5.1 Optimization of the reverse transcription of RNA extracted from Atlantic salmon macrophages.

Optimization of reverse transcription conditions was performed using a gradient thermocycler, a range of reverse transcription duration and temperature variables were used to generate cDNA from a sample originating from IPNV-inoculated macrophages. Optimization was performed in accordance with the recommendations contained in the TaqMan® Reverse Transcription reagents protocol. Thus, reaction temperatures in the range of 42°C to 52°C were tested together with reaction durations ranging from 20 to 60 minutes (section 4.2.3.1). The cDNA produced was then amplified using the primers and probes for the Mx and ELF-1 genes. The optimal conditions for reverse transcription for ELF and Mx were chosen by selected by the samples that amplified at the earliest Ct value as shown in Figure 9 and Figure 10. The conditions considered to represent optimal amplification parameters were 60 minutes at 46.4°C (Table 4). These experimental parameters were subsequently used for reverse transcription in all RT-PCR experiments performed in this study.

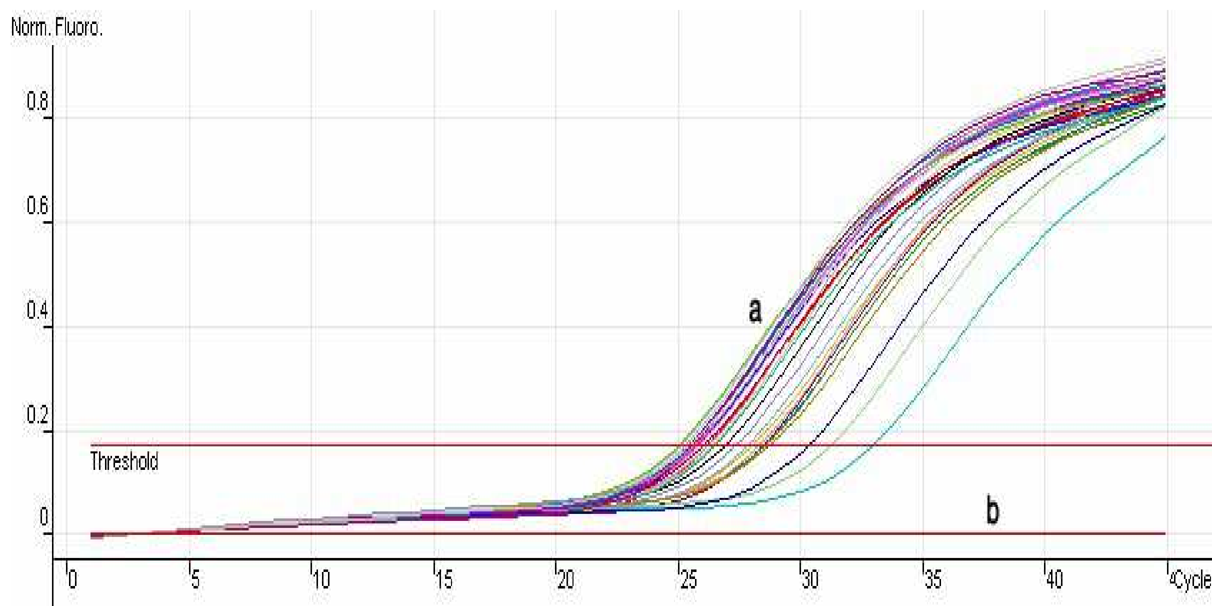


Figure 9: Real time RT-PCR amplification using ELF-1 specific primers and ELF-1 probe to amplify a cDNA template derived from Poly I:C stimulated macrophages. The following variables in the reverse transcription step were investigated: reaction temperatures, 42°C, 46.4°C and 52°C; reaction duration 20, 30, 40, 50, and 60 minutes. The sample showing the earliest Ct value corresponded to reverse transcription condition 60 minutes at 46.4°C (a). No template controls and RT-minus controls were incorporated into amplification runs (b).

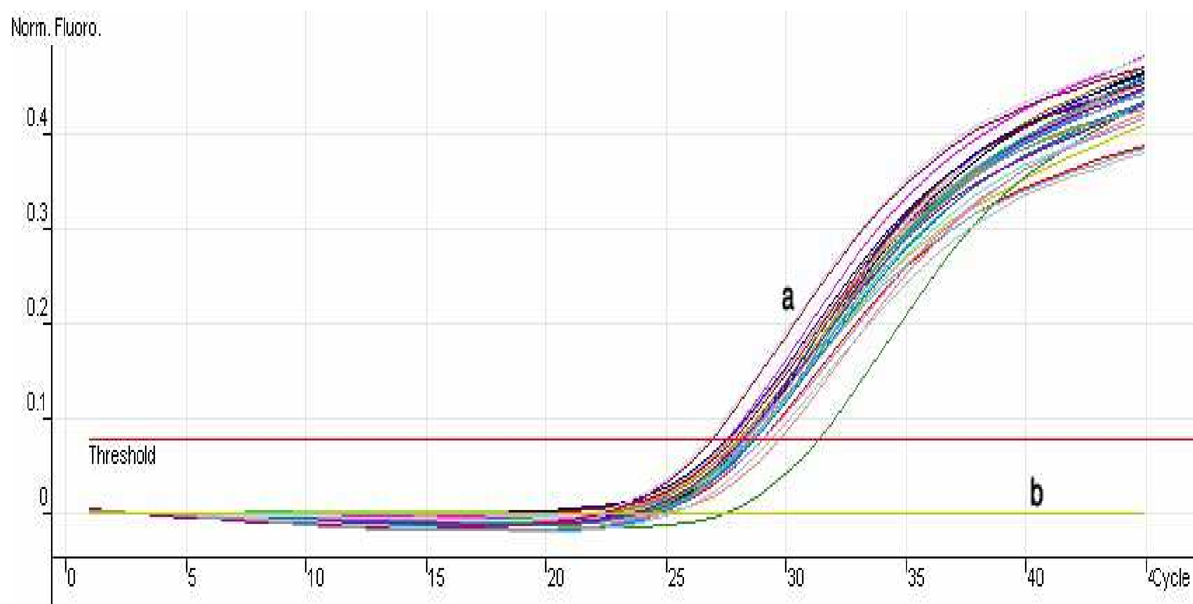


Figure 10: Real time RT-PCR amplification using Mx specific primers and Mx probe to amplify a cDNA template derived from Poly I:C stimulated macrophages. The following variables in the reverse transcription step were investigated: reaction temperatures, 42°C, 46.4°C and 52°C; reaction duration 20, 30, 40, 50, and 60 minutes. The sample showing the earliest ct value corresponded to reverse transcription condition 60 minutes at 46.4°C (a). No template controls and RT-minus controls were incorporated into amplification runs (b).

Table 4: Ct values corresponding to the real time RT-PCR amplification using both ELF-1 and Mx specific primers along with ELF-1 and Mx specific probes to amplify a cDNA template derived from Poly I:C stimulated macrophages.

ELF-1 sample	Conditions	Ct	Average Ct		Mx sample	Name	Ct	Average Ct
1	20 mins 42	26.31			1	20 mins 42	29.14	
2	20 mins 42	28.15	27.23		2	20 mins 42	28.7	28.92
3	20 mins 46.4	25.85			3	20 mins 46.4	28.33	
4	20 mins 46.4	28.56	27.205		4	20 mins 46.4	28.23	28.28
5	20 mins 52	25.97			5	20 mins 52	28.53	
6	20 mins 52	25.48	25.725		6	20 mins 52	28.83	28.68
7	30 mins 42	28.63			7	30 mins 42	28.66	
8	30 mins 42	28.42	28.525		8	30 mins 42	29.84	29.25
9	30 mins 46.4	28.66			9	30 mins 46.4	31.45	
10	30 mins 46.4	25.83	27.245		10	30 mins 46.4	27.7	29.575
11	30 mins 52	26.98			11	30 mins 52	28.43	
12	30 mins 52	33.04	30.01		12	30 mins 52	28.64	28.535
13	40 mins 42	26.5			13	40 mins 42	27.75	
14	40 mins 42	31.31	28.905		14	40 mins 42	28.26	28.005
15	40 mins 46.4	25.18			15	40 mins 46.4	28.72	
16	40 mins 46.4	27.95	26.565		16	40 mins 46.4	28.5	28.61
17	40 mins 52	27.45			17	40 mins 52	28.46	
18	40 mins 52	25.68	26.565		18	40 mins 52	29.17	28.815
19	50 mins 42	26.34			19	50 mins 42	27.79	
20	50 mins 42	26.15	26.245		20	50 mins 42	27.95	27.87
21	50 mins 46.4	28.86			21	50 mins 46.4	28.19	
22	50 mins 46.4	25.08	26.97		22	50 mins 46.4	28.35	28.27
23	50 mins 52	26.59			23	50 mins 52	28.7	
24	50 mins 52	25.6	26.095		24	50 mins 52	28.83	28.765
25	60 mins 42	30.38			25	60 mins 42	27.7	
26	60 mins 42	25.49	27.935		26	60 mins 42	28.33	28.015
27	60 mins 46.4	25.68			27	60 mins 46.4	27.04	
28	60 mins 46.4	25.51	25.595		28	60 mins 46.4	26.87	26.955
29	60 mins 52	25.81			29	60 mins 52	29.49	
30	60 mins 52	25.75	25.78		30	60 mins 52	28.68	29.085
31	RT-	0			31	RT-	0	
32	RT-	0	0		32	RT-	0	0
33	NTC	0			33	NTC	0	
34	NTC	0	0		34	NTC	0	0

4.5.2 Sensitivity of real time RT-PCR assays

The amplification efficiencies for all the targets investigated in this thesis were estimated by amplifying a 10-fold dilution series of the gene target (Figure 11, Figure 13, Figure 15 and Figure 17). Using the slope of the resulting trend line the PCR efficiencies of the genes were calculated through the Rotor-Gene software (Figure 12, Figure 14, Figure 16, Figure 18). Table 5 shows that all of the efficiencies for the targets in this study were above 85%.

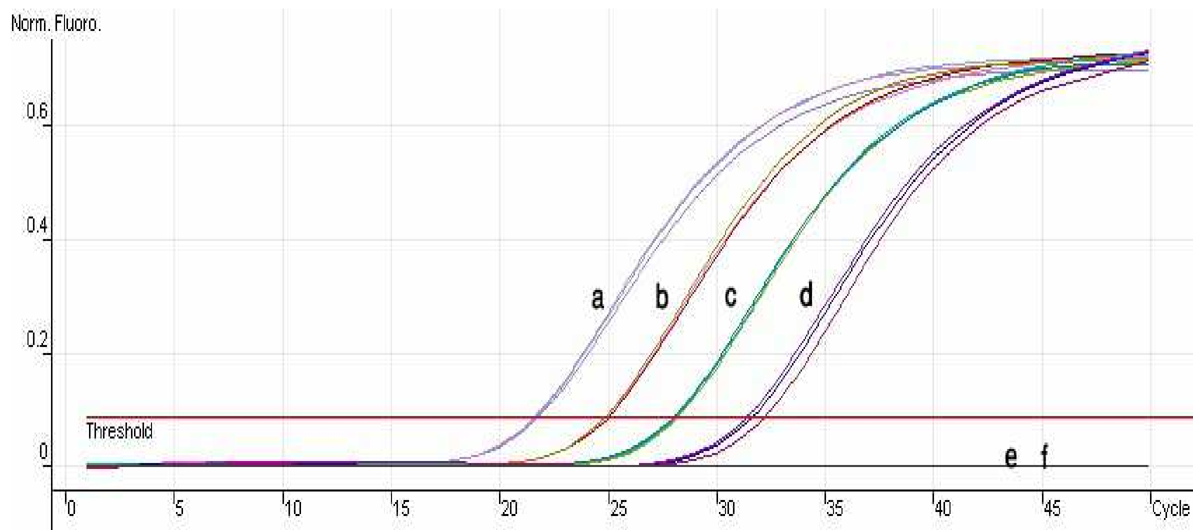


Figure 11: Real time RT-PCR amplification using ELF-1 specific primers and ELF-1 probe to amplify a ten-fold dilution series of a cDNA template derived from Poly I:C stimulated macrophages. Amplifications were performed in triplicate. Target dilutions tested were 10^0 (a), 10^1 (b), 10^2 (c), 10^3 (d), 10^4 (e), No template controls and RT-minus controls were incorporated into amplification runs (f).

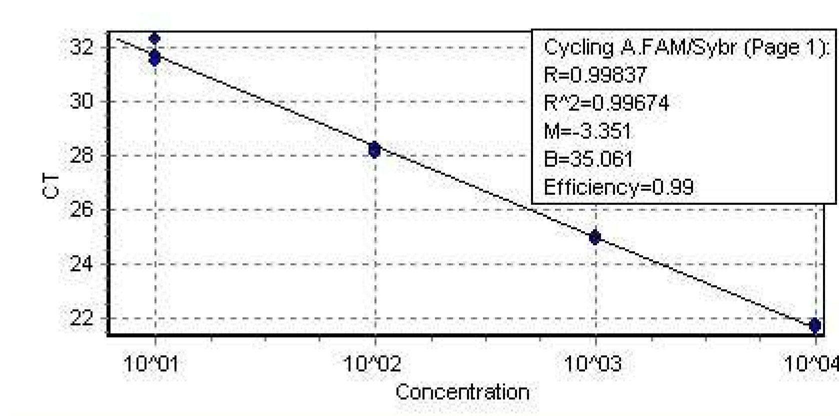


Figure 12: Standard curve obtained from amplification using ELF-1 specific primers and ELF-1 probe produced using the Rotor-Gene software. Amplification efficiencies were calculated according to the equation: E = 10[$^{-1}$/slope].

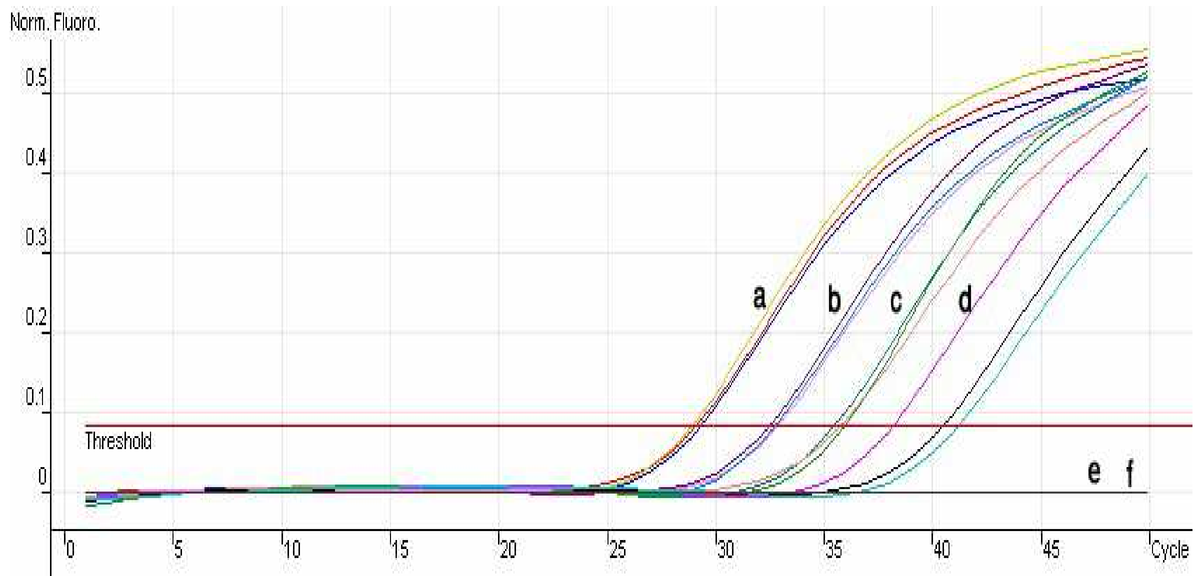


Figure 13: Real time RT-PCR amplification using Mx-specific primers and Mx probe to amplify a ten-fold dilution series of a cDNA template derived from Poly I:C stimulated macrophages. Amplifications were performed in triplicate. Target dilutions tested were 10^0 (a), 10^1 (b), 10^2 (c), 10^3 (d), 10^4 (e), No template controls and RT-minus controls were incorporated into amplification runs (f).

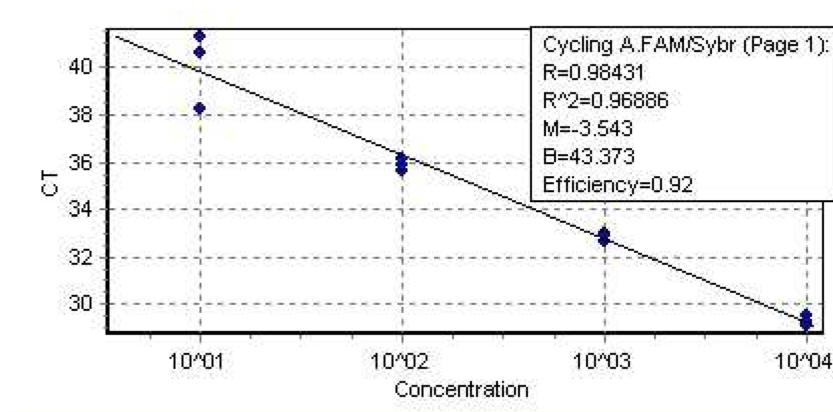


Figure 14: Standard curve obtained from amplification using Mx-specific primers and Mx-specific probe produced using the Rotor-Gene software. Amplification efficiencies were calculated according to the equation: E = 10[$^{-1}$/slope].

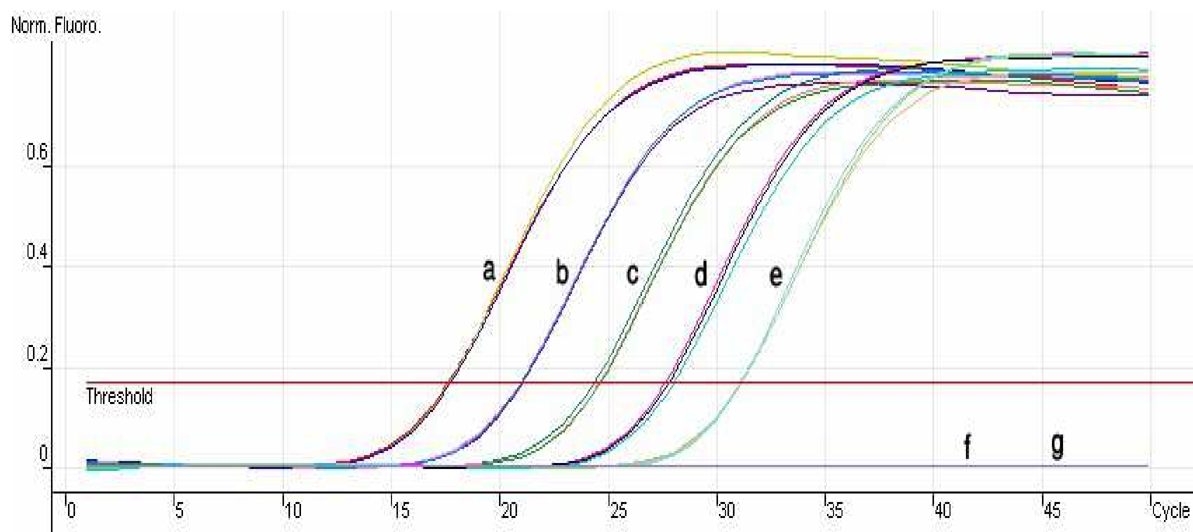


Figure 15: Real time RT-PCR amplification using IPNV-specific primers and IPNV probe to amplify a ten fold dilution series of cDNA derived from IPNV-infected macrophages. Amplifications were performed in triplicate. Target dilutions tested were 10^0 (a), 10^1 (b), 10^2 (c), 10^3 (d), 10^4 (e), 10^5 (f), No template controls and RT-minus controls were incorporated into amplification runs (g).

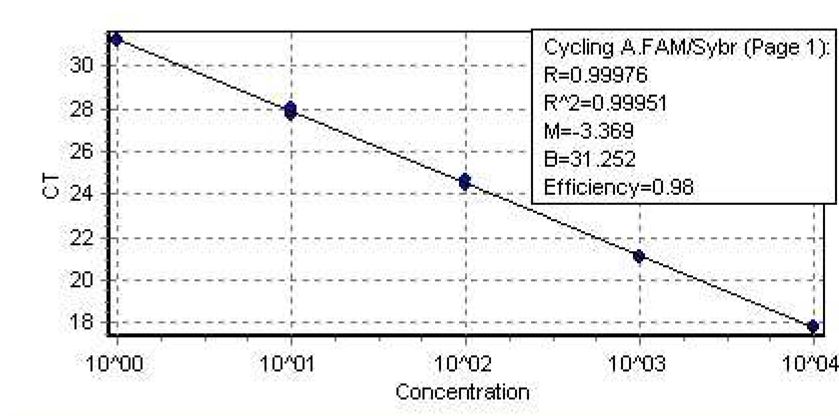


Figure 16: Standard curve obtained from amplification using IPNV-specific primers and probes produced using the Rotor-Gene software. Amplification efficiencies were calculated according to the equation: E = 10[$^{-1}$/slope].

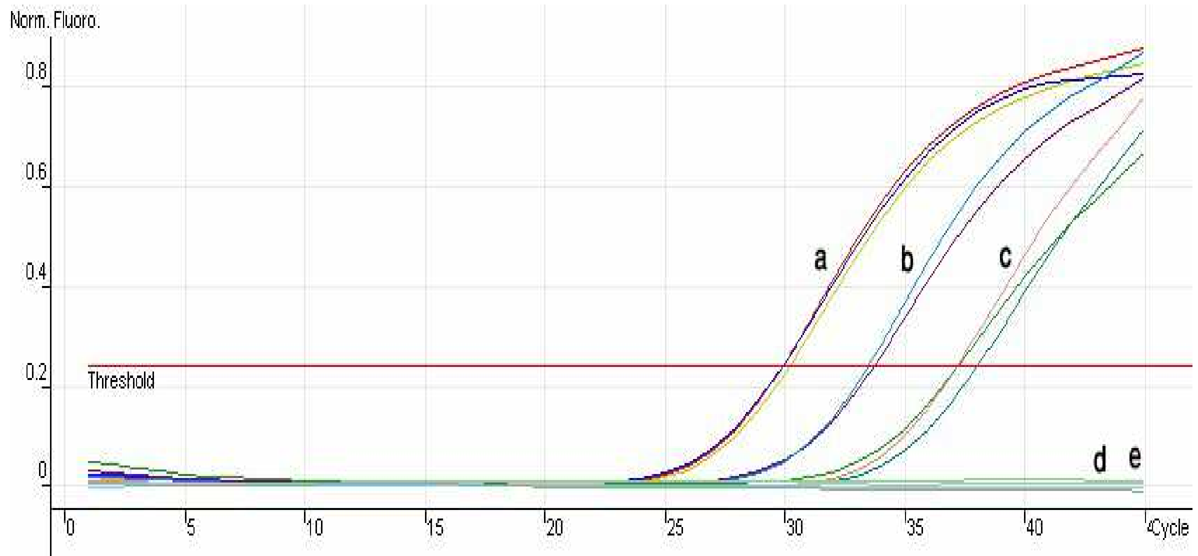


Figure 17: Real time RT-PCR amplification using IFN-specific primers and IFN probe to amplify a ten fold dilution series of a cDNA derived from Poly I:C stimulated macrophages. Amplifications were performed in triplicate. Target dilutions tested were 10^0 (a), 10^1 (b), 10^2 (c), 10^3 (d), No template controls and RT-minus controls were incorporated into amplification runs (e).

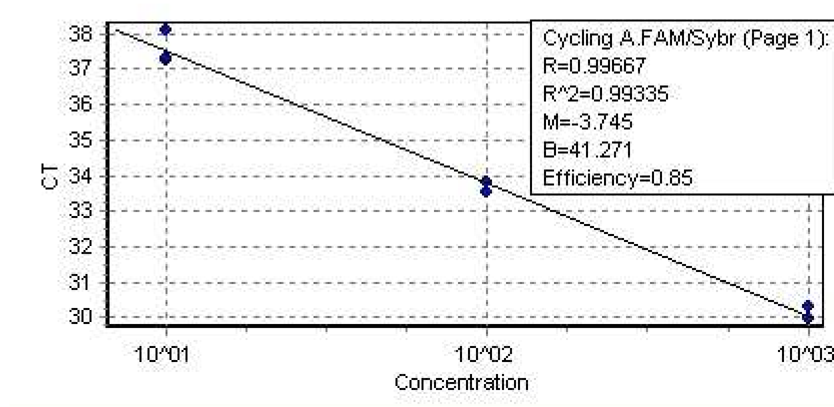


Figure 18: Standard curve obtained from amplification using IFN-specific primers and probes produced using the Rotor-Gene software. Amplification efficiencies were calculated according to the equation: E = 10[$^{-1}$/slope].

Table 5: Amplification efficiencies and R values for the primers and probes used in this study

Target	R Value	Efficiency
ELF-1	0.99	0.99
Mx	0.98	0.92
IPNV	0.99	0.98
IFN	0.99	0.85

4.6 Discussion

4.6.1 Optimization of first strand cDNA synthesis

The RT step is the source of most of the variability in a quantitative RT-PCR experiment (Freeman *et al*., 1999). Therefore, the conditions for the first strand synthesis of cDNA from the extracted RNA were optimised. Random hexamers were selected for the production of cDNA from RNA extracted from macrophages. Random hexamer primers contain all possible nucleotide sequences of a 6 base oligonucleotide, and bind to multiple points on target nucleic acid. They are particularly useful for targets with significant secondary structure such as the IPNV genome. The products of RT reactions primed by random hexamers can be split for use in several PCR reactions each utilising a different gene-specific primer pair. This method maximises the number of genes that can be assayed from a small sample (Freeman *et al*., 1999). As the expression of all targets will be from the same cDNA, it was therefore necessary to use first strand primers that will amplify total RNA. As IPNV does not have an poly-A tail it was not possible to use oligo d(T)16 primers and therefore random hexamers were the most suitable choice for first strand synthesis. It is necessary to acknowledge that the different priming methods used to generate cDNA differ significantly with respect to specificity and cDNA yield and variety (Bustin *et al.,* 2005). However as the cDNA is generated in the same way and the RT conditions are the same for all of the targets studied these problems should not impact on the work performed in this thesis. RNA can exhibit significant secondary structure that affects the ability of the RNA-dependant DNA polymerase (reverse transcriptase, RT) to generate transcripts (Bustin,

2000). The 10 minute denaturation of the total RNA and primers prior to first strand synthesis was incorporated in response to the findings of Lopez-Lastra *et al.,* (1994). These workers concluded that first strand synthesis is the most important reaction step with respect to amplification of IPNV, due to its double stranded genome. The use of a two step RT-PCR method, i.e. separating the RT and the PCR steps, as compared to single step RT-PCR provides a considerable advantage in that it produces a cDNA pool or library which can be kept indefinitely (Bustin, 2000, Peters *et al*., 2004), therefore other mRNA targets can be quantified with relative ease provided that specific primers and probes are available.

4.6.2 Selection of the housekeeping gene for use in relative quantitation by real-time qRT-PCR

An ideal housekeeping gene should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by experimental treatment (Zhang *et al.,* 2005). Studies have shown that expression of some of the commonly used housekeeping genes can vary under experimental conditions (Radonic *et al.,* 2004;Vandesompele *et al.*, 2002; Schmittgen and Zakrajsek 2000). It is the opinion of Radonic *et al.,* (2004), that whilst it seems unreasonable that the transcription of any gene in a living cell is absolutely resistant to cell cycle fluctuations or nutrient status, it is important to identify candidate genes that are least minimally regulated during individual experiments allowing the accuracy of RNA transcription analysis that real-time PCR offers. Unfortunately, due to constraints imposed by funding and time, it was not possible to perform efficacy studies on internal control genes. Therefore, a housekeeping gene (ELF-1) was obtained from FRS Marine Laboratory, Aberdeen, which has successfully been used in

real time RT-PCR studies of fish immune gene expression (McBeath *et al*., 2006); (Collet *et al.,* 2007); (Lockhart *et al.,* 2007).

4.6.3 Real-time quantitative PCR for IFN, Mx, IPNV and ELF-1

In this study MGB probes were used to monitor product accumulation in real-time. The choice of MGB probes was based on their successful use at FRS laboratory, Aberdeen. Probes of all types are more expensive than reporter dyes such as SYBR Green I, but they permit sequence-specific detection, minimising false positive reactions due to detection of non-specific amplification products and primer dimers (Peters *et al.,* 2004). The advantage of these MGB probes over the SYBR Green I DNA binding probes is based on the specific binding between probe and target that is required to generate a signal, unlike SYBR Green I, which binds to any double stranded DNA produced during amplification. Hydrolysis probes achieve fluorescence by separating the fluorophore and quencher through the 5' to 3' endonuclease activity of Taq polymerase during primer extension (Steuerwald *et al.,* 1999). The use of probes with modified chemistry, such as those containing high-affinity DNA minor-groove binding moieties has further improved quantitative PCR sensitivity by increasing both probe hybridization and signal-to-noise ratios (Grace *et al.,* 2003). The probes used in this study were synthesised with 3' MGB modifications, which raises the effective melting temperature (Tm) of the probe, thereby enabling the probe to be significantly shorter (Ginzinger, 2002). Kutyavin *et al.,* (2000) have shown that MGB probes with their shorter sequence lengths give better sequence specificity and lower fluorescent background in comparison with conventional TaqMan probes. Hybridization probes provide a very high sensitivity due to low background fluorescence levels, however,

as previously mentioned, there is a high cost for synthesis of each new gene specific probe (Kreuzer *et al.,* 1999). As a lot of the work done in this study involved an investigation of changes in expression of targets expressed at a relatively low level, the use of these highly sensitive and specific MGB probes was important. The use such specific amplification chemistry permitted detection of all targets studied over a concentration of several log_{10}. The IFN gene, which was expected to be the most difficult to detect target due to its very confined and short lived expression, could only be detected over a concentration range of 2 log_{10} (Figure 17). PCR in this study was performed using a Universal PCR mastermix made by Applied Biosystems. According to the manufacturer's notes it is not necessary to perform titration experiments to obtain optimal concentrations of reaction components, such as magnesium chloride, as their master mix is specifically designed to provide optimal performance for TaqMan assays that use cDNA as a substrate under universal cycling conditions. The amplification efficiency of a given gene can be estimated by amplifying a 10-fold dilution series of the gene target, and by plotting the Ct values obtained as a function of the log_{10} of target concentration. The slope of the resulting trend line will be a function of the PCR efficiency (Ginzinger, 2002). The results of the present study show that RT-PCR assays for all of the genes studied had good efficiency values all above 90% apart from IFN which had an efficiency of only 85% (Table 5). According to Ginzinger (2002) using the relative quantitation method requires that the PCR efficiencies of all genes be similar and preferably at or above 90%. It was decided that as only one gene had efficiency below 90% it was still possible to use the relative quantitation method as there are relative mathematical methods available that incorporate amplification efficiency correction into their calculations and additionally do not require the reaction efficiencies to be similar.

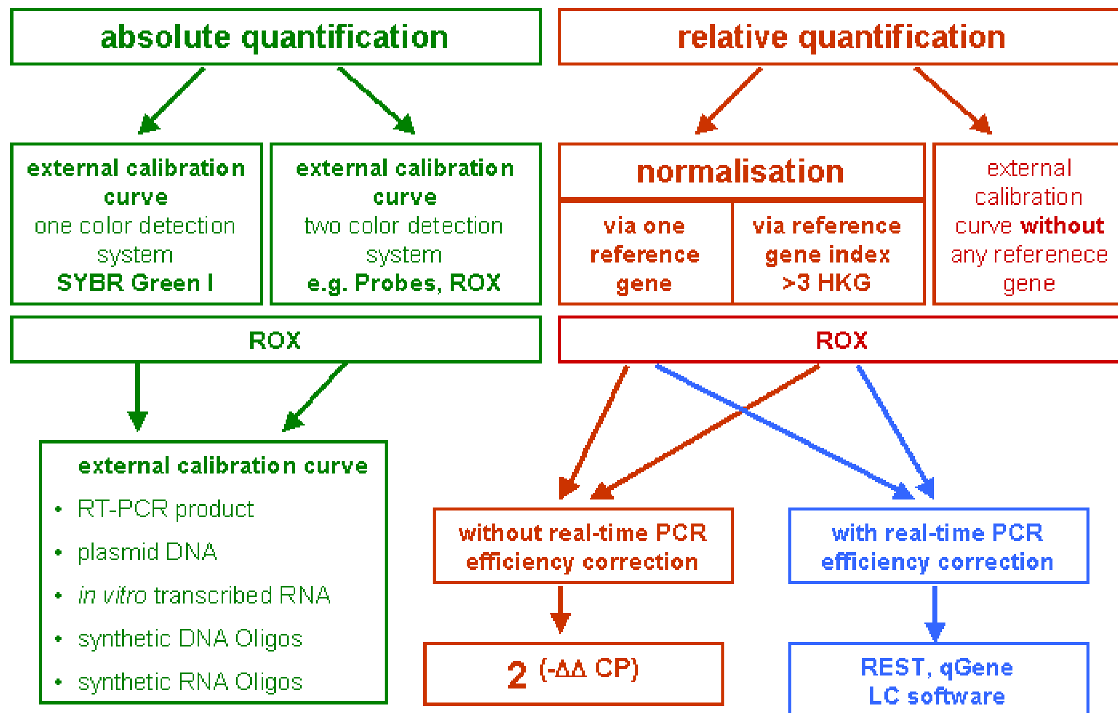
Other studies have successfully utilised RT-PCR procedures with efficiencies as low as 70% for quantitation (Jorgensen *et al.,* 2006).

4.6.4 Selection of method for quantification in real-time RT-PCR

Two different methods of analyzing data from real-time, quantitative PCR exist: absolute quantification and relative quantification (Livak and Schmittgen, 2001). Relative quantification is based on the relative expression of a target gene versus a reference gene (Pfaffl, 2001) whilst absolute quantification uses serially diluted standards of known concentrations to generate a standard curve (Wong and Mendrano, 2005). The advantage of absolute quantification is that it is easier to compare expression data between different days and laboratories, because the calibration curve provides a fixed reference point (Pfaffl, 2002). However, absolute quantification requires a number of extra conditions and treatments that relative quantification does not (Freeman *et al.,* 1999). It was decided that relative quantification would be adequate for analysis of gene expression associated with *in vitro* activity, as the main emphasis would be on patterns of expression over time in relation to stimuli rather than the exact copy numbers of specific genes at specific time points. The decision regarding the choice of method used to analyse the results from this study was made in reference to Table 6 from Wong and Medrano (2005) which compares the various relative quantitation mathematical methods available and Figure 19, which is a flow diagram, which recommends appropriate methods concerning the nature of the results being evaluated.

Table 6: Characteristics of various relative quantitation methods

Methods	**Amplification Efficiency Correction**	**Amplification Efficiency Calculation**	**Amplification Efficiency Assumptions**	**Automated Excel-Based Program**
Standard Curve (Livak, 1997).	No	Standard curve	No experimental sample variation	No
Comparative C_t ($2^{-\Delta\Delta Ct}$) (Livak,Schmittgen, 2001)	Yes	Standard curve	Reference = target	No
Pfaffl (Pfaffl, 2001)	Yes	Standard curve	Sample = control	REST
Q-Gene (Muller, Janovjak, Miserez, Dobbie, 2002).	Yes	Standard curve	Sample = control	Q-Gene
Gentle (Gentle, Anastasopoulos, McBrien, 2001).	Yes	Raw data	Researcher defines log-linear phase	No
Lui and Saint (Lui & Saint, 2002).	Yes	Raw data	Reference and target genes can have different target efficiencies	No
DART-PCR (Peirson, Butler, Foster, 2003).	Yes	Raw data	Statistically defined log-linear phase	DART-PCR



Key

ROX = Reporter dye associated with probe/beacon

HKG = Housekeeping gene

Figure 19: Flow diagram for the selection of a quantitative mathematical method (www.wzw.tum.de/gene-quantification)

The Pfaffl method for relative quantification was chosen for the following reasons. As the amplification reaction efficiencies were not all similar the method required amplification efficiency correction, a function which was not provided by the standard curve method. According to Table 6 whilst the Comparative Ct ($2^{-\Delta\Delta Ct}$) method includes a correction for non-ideal reaction efficiencies, it has been shown that when using this method the amplification efficiencies of the target gene and reference genes must be approximately equal, otherwise differences in efficiencies will generate errors (Livak and Schmittgen, 2001; Lui and Saint, 2002). Therefore, as Figure 19 shows, this method is not suitable for a test which requires efficiency correction, for this reason the Pfaffl mathematical model was used in the present study. REST is a software tool to estimate up and down-regulation for gene expression studies. The purpose of REST is to determine whether there is a significant difference between samples and controls, while taking into account issues of reaction efficiency and reference gene normalisation. REST uses the Pfaffl mathematical model to generate relative expression ratios, and subsequently the significance of the results are investigated using a randomisation test. Unfortunately, from talking to representatives for the REST software, it is not possible to use REST for time course experiments. Whilst the developers of REST recognize this as a major limitation of the software, it is however still possible to analyze the results of any experiment with the Pfaffl mathematical model without the aid of the REST programme. The Pfaffl mathematical model combines gene quantification and normalization into a single calculation (Wong and Mendrano, 2005). According to Pfaffl (2001), this method has taken into consideration the mathematics of the Comparative Ct ($2^{-\Delta\Delta Ct}$) method in order to better understand the mode of Ct data analysis and for a more reliable and exact gene expression. The Pfaffl method is simply calculated

from the real-time PCR efficiencies and the cycle threshold (Ct) deviation of an unknown sample versus a control. This incorporation of the amplification efficiencies of the target and reference (normalization) genes allows for the correction of differences between two assays (Wong and Mendrano, 2005). Table 6 shows that in essence the Q-Gene method has the same characteristics as the Pfaffl method, however on the basis of a literature search the Pfaffl method appeared to be a more widely used method and it was for this reason that the Pfaffl method was selected over the Q-Gene method. It is the opinion of Pfaffl, (2001) that this mathematical method is an ideal and simple tool for the verification of amplification results without the need for more complex and time consuming quantification models based on calibration curves. The Pfaffl method uses the following equation as outlined in Figure 20.

$$\text{ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (control-treated)}}{(E_{ref})^{\Delta Ct\ ref\ (control-treated)}}$$

Key

E = Amplification efficiency of gene

Ct = Cycle threshold

Figure 20: Pfaffl calculation for the relative expression in real time PCR (from Pfaffl *et al*., 2002).

4.6.5 Explanation of components of Pfaffl calculation

The Pfaffl mathematical model for the calculation of relative expression in real-time PCR. The relative expression ratio of a target gene is calculated based on its real-time PCR efficiencies (E) and the Ct deviation (Δ) of an unknown sample versus a control ($_{control-sample}$). Prior to treatments, RNA was extracted from macrophages to identify the normal expression values for all of the targets investigated in the experiment. This extraction was termed "time 0" and the values were incorporated within the Pfaffl relative quantification calculation as the "control".

4.7 Conclusion

This chapter is concerned with optimization of the conditions for amplification and subsequent detection of target mRNA obtained from Atlantic salmon macrophages. The results from chapters 3 and 4 can be used to successfully develop an *in vitro* infectious experimental protocol. Alongside the optimised real time RT-PCR method, this will allow experiments to be conducted whose results will be subject to quantitative analysis to advance understanding of how IPNV affects the innate immune response within Atlantic salmon head kidney macrophages.

Chapter 5 - Innate immune responses in Atlantic salmon macrophages infected with IPNV: induction and effect of immunostimulants

5.1 Introduction

Mammalian cells possess diverse defence mechanisms against viral infection, with one of the most important of these being an antiviral state induced by type I IFN (Collet *et al.,* 2007). The mammalian IFN system has been characterised in detail at the molecular level, however in fish, this system is poorly understood (Johansen *et al.,* 2004). Advancing current knowledge of the non-specific antiviral defence mechanisms of Atlantic salmon might help to explain this species' susceptibility to IPNV (Jensen and Robertsen, 2002). In particular, innate immune responses to IPNV within macrophages are poorly characterised. Knowledge of macrophage immune responses to IPNV could facilitate the design of vaccination strategies to counter IPNV, and breeding programmes aimed at the production of IPN-resistant fish.

Immunostimulants are of potential importance for the control of fish diseases and may thus be useful in aquaculture of marine fish (Sakai, 1999). The use of immunostimulants in fish culture offers a wide range of attractive methods for inducing and boosting protection against infectious diseases (Anderson, 1992). The susceptibility of Atlantic salmon to IPN is greatest in juvenile life cycle stages and in smolts shortly after seawater transfer (Rønneseth *et al.,* 2006). It is the opinion of Anderson (1992) that in cases where disease can be predicted, losses may be reduced by elevating non-specific

defence mechanisms through the use of immunostimulants to prevent losses from diseases. The non-specific immune system has evolved towards recognition of structurally conserved microbial polymers such as fungal cell wall ß-glucans, bacterial lipopolysaccharide (LPS), bacterial DNA and double-stranded RNA (dsRNA) (Robertsen, 1999). In the last decade, many studies have focused on the use of immunostimulants in fish farming as alternatives or supplements to vaccination or chemotherapeutants (Salinas *et al.,* 2004). Currently used fish immunostimulants, inclusive of both synthetic chemicals and biological substances have been reviewed Sakai (1999); (Bricknell and Dalmo, 2005), however, there are no records of any of them having an effect towards IPNV. Most of these studies have focused on the protection against bacterial pathogens and/or non-specific immune parameters such as phagocytic, complement or lysozyme activities whilst studies on increased protection against viral infection are scarce. Thus, the ability of the immunostimulants macrophage activation factor (MAF), lipopolysaccharide (LPS) and glucan to protect macrophages from infection with IPNV will be investigated in this chapter.

The aims of this chapter are:

a) To determine whether IPNV infects Atlantic salmon head kidney macrophages *in vitro* and if so is it able to replicate? (i.e. to a level greater than that contained in the inoculums used to infect macrophages).

b) To characterise the IFN response of Atlantic salmon macrophages to IPNV: specifically to determine whether IPNV induces an Mx response, how this

compares to that induced by IFN, and whether IFN affects the replication and persistence of IPNV within macrophages.

c) To investigate the effects of immunostimulants on the macrophage IFN response to IPNV. The effects of glucan, MAF and LPS on IFN and Mx expression will be determined in IPNV-infected macrophages and uninfected controls. The effects of these immunostimulants on IPNV replication will also be studied.

d) Preparations of IFN are required to perform the experimental work outlined above, thus the chapter also includes a description of the methodology used to produce IFN from Atlantic salmon head kidney macrophages.

5.2 Materials and Methods

5.2.1 Production of IFN-preparations from Atlantic salmon macrophages

Head kidneys were obtained from Atlantic salmon as outlined in section 2.2.2. Macrophages were isolated on 51% percoll as described in section 2.2.3 and maintained in culture as described in section 2.2.5. After 24 hours the macrophages were washed once in L-15 medium and then cultured in the same medium containing Poly I:C as described in section 4.2.2, whilst control wells received only L-15 medium in place of Poly I:C. After 12 hours, the cells were washed three times with L-15 medium and then cultured in fresh L-15 supplemented with 50 units ml^{-1} penicillin, 50μg ml^{-1} streptomycin and 5% FCS, at 15°C. The culture medium was harvested after 48 hours, centrifuged for

10 minutes at 400 x g and then the supernatant stored in aliquots at -70ºC until assayed for IFN activity.

5.2.2 Efficacy of IFN-preparations in stimulation of Mx response

Detection of IFN-like activity in the macrophage supernatants was performed using RTG-P1 cells, obtained from the Marine laboratory, Aberdeen. RTG-P1 cells are transfected with a luciferase reporter gene under the control of the Mx promoter gene (Collet *et al.,* 2004), thus the transcriptional activity of the Mx promoter can be readily quantified through luciferase assays. RTG-P1 cells were cultured in L-15 medium supplemented with 10% FCS and 200µg ml^{-1} Neomycin (Sigma) at 20ºC. RTG-P1 cells were grown to 100% confluence and seeded onto 24 well plates and incubated overnight at 20ºC. The following day 500µl of the macrophage supernatant was added to the RTG-P1 cells in triplicate wells and incubated for 48 hours at 20ºC. Controls received supernatants originating from untreated macrophages and L-15 medium which had no previous contact with macrophages. The supernatants were removed and 100µl of luciferase substrate (Steady-Glo, Promega) was added to the wells for 2 minutes and the resulting cell lysate was stored at -70ºC prior to testing for luciferase expression.

5.2.3 Luciferase assay

Luciferase activity was measured by a luciferin-ATP assay and photon emission was measured using a MLX luminometer (Dynex Tecnology) by recording the integrated sum of light emitted for 10 seconds and expressed as relative light units (RLU). Results are expressed as the mean (N=3) ± SD of the luciferase activity expressed by RTG-P1 cells.

Data was analysed on the MINITAB software package by a one-way ANOVA, and a Tukey's test was used perform comparisons between the luciferase activating properties of the IFN supernatants. Differences were considered statistically significant when $P<0.05$.

5.2.4 Production of MAF containing supernatants by salmon leucocytes

Head kidneys were obtained from Atlantic salmon as outlined in section 2.2.2. The head kidney cell suspension was layered over 51% percoll gradients and centrifuged at 400 x g for 30 minutes at 4°C. The leucocyte fraction was removed from the percoll-medium interface and then washed in serum free L-15 medium. The leucocytes were adjusted to a concentration of 5 x 10^{-6} live cells/ml L-15 medium containing 5 x 10^{-5} M 2-mercaptoethanol (2ME). 25cm^2 tissue culture flasks were then seeded with 5ml aliquots of the leucocyte suspension and pulsed for 3 hours at 15°C with concanavalin A (ConA); (Sigma) 10µg/ml^{-1} and 5ng/ml^{-1} phorbol myristate acetate (PMA); (Sigma). Cells were then washed three times with phosphate buffered saline to remove any residual ConA and PMA and cultured in L-15 medium containing 10% FCS at 15ºC. Immediately after addition of the medium, 300µl was removed from each flask and frozen to provide a control to show that any observed effects result from the MAF supernatant and not from any remaining residual Con A or PMA. After 48 hours, the supernatants were harvested, centrifuged and stored at -70ºC until use.

5.2.5 Measurement of MAF activity

Head kidneys were obtained from Atlantic salmon as outlined in section 2.2.2. Macrophages were isolated on 51% percoll as described in section 2.2.3 and maintained in culture as described in section 2.2.5. The cell suspension was seeded into 96 well culture plates, adding 100µl to each well. Unattached cells were washed off after 3 hours and the macrophage monolayers were maintained in L-15 medium supplemented with 50 units ml^{-1} penicillin, 50µg ml^{-1} streptomycin and 5% FCS overnight. The following day the culture medium was removed and replaced with MAF-containing supernatants diluted 1:4 and 1:8 in L-15 medium, 10% FCS and 5×10^{-5} M 2ME. These macrophage monolayers were incubated with the MAF supernatants for 48 hours. After incubation, the macrophage activating properties of the supernatants were evaluated by respiratory burst assays. Triplicate wells in 96 well plates of macrophages were incubated with 100µl of L-15 medium containing 1mg/ml nitroblue tetrazolium (NBT); (Sigma) and 1µl/ml PMA for 30 minutes whilst control wells were incubated with 100µl of L-15 medium containing 1mg/ml NBT. The reaction was stopped by fixing the macrophages with 100% methanol, followed by washing with 70% methanol to remove any extracellular formazan. The reduced intracellular formazan was solubilised in 120µl 2M potassium hydroxide (KOH) and 140µl dimethyl sulfoxide (DMSO). The plates were then read at a wavelength of 620nm using a plate reader.

5.2.6 Lipopolysaccharide (LPS) and glucan

LPS was kindly provided by Remi Gratacap, Department of Aquaculture, Stirling University, and was extracted from the marine pathogen *Vibrio anguillarum* using butan-1-ol (Gratacap pers comm.). Laminarin was purchased from Sigma in order to make the glucan supernatants. Laminarin and LPS were resuspended in L-15 medium to 100µg ml^{-1} and 10µg ml^{-1} respectively.

5.2.7 Isolation of macrophages to obtain macrophage monolayers for *in vitro* experiments

Head kidneys were obtained from Atlantic salmon (section 2.2.2) and macrophages were isolated on 51% percoll as described in 2.2.4. Macrophages were seeded into 24 well plates at concentrations of 2 x 10^{-7} cells ml^{-1} and maintained at 15°C as described in section 2.2.5.

5.2.8 *In vitro* virus infection in isolated macrophages

In this chapter, two experiments were performed. Firstly, a trial infection of Atlantic salmon head kidney macrophages with IPNV was conducted. As this thesis is concerned with the interaction between IPNV and the macrophage innate immune response, it was necessary to demonstrate that Atlantic salmon macrophages could be infected with IPNV (i.e. – that the level of IPNV in infected macrophages increases over that contained in the inoculum used to infect these cells). To conduct the experiment, macrophage monolayers were processed following the guidelines as set out below regarding the positive control

group and negative control group. The IPNV levels in macrophages were examined by conducting RNA extraction on the macrophage monolayers immediately after infection (time 0) and at days 1,3,5,7 and 9 days post-infection, following the modified protocol as described in section 3.2.5. In the second experiment the ability of a number of different immunostimulants to protect macrophages from infection with IPNV were investigated. To conduct this experiment the macrophage monolayers were processed in triplicate using one of the following ten treatments at 15°C. Inoculation of virus for the treatment groups was performed as described in section 2.2.7.

a) IFN-stimulated group. Macrophages were incubated for 24 hours with IFN supernatants only.

b) IFN-stimulated and IPNV-infected group. Macrophages were incubated for 24 hours with IFN supernatants prior to infection with IPNV at an MOI of 1.

c) LPS-stimulated group. Macrophages were incubated for 24 hours with L-15 medium, supplemented with 50 units ml^{-1} penicillin, 50μg ml^{-1} streptomycin and 5% FCS, containing LPS at 10μg ml^{-1} only

d) LPS-stimulated and IPNV-infected group. Macrophages were incubated for 24 hours with L-15, supplemented with 50 units ml^{-1} penicillin, 50μg ml^{-1} streptomycin and 5% FCS, containing LPS at 10μg ml^{-1} prior to infection with IPNV at an MOI of 1.

e) MAF-stimulated group. Macrophages were incubated for 24 hours with MAF diluted 1:8 in L- 15 medium, supplemented with 50 units ml^{-1} penicillin, 50μg ml^{-1} streptomycin and 5% FCS only.

f) MAF-stimulated and IPNV-infected group. Macrophages were incubated for 24 hours with MAF diluted 1:8 in L-15 medium, supplemented with 50 units ml^{-1} penicillin, 50μg ml^{-1} streptomycin and 5% FCS, prior to infection with IPNV at an MOI of 1.

g) Glucan-stimulated group. Macrophages were incubated for 24 hours with L-15 medium, supplemented with 50 units ml^{-1} penicillin, 50μg ml^{-1} streptomycin and 5% FCS, containing glucan at 100μg ml^{-1} only.

h) Glucan-stimulated and IPNV-infected group. Macrophages were incubated for 24 hours with L-15 medium, supplemented with 50 units ml^{-1} penicillin, 50μg ml^{-1} streptomycin and 5% FCS, containing glucan at 100μg ml^{-1} prior to infection with IPNV at an MOI of 1.

i) Positive control group. Macrophages were infected with IPNV at an MOI of 1.

j) Negative control group. Macrophages were maintained in L-15 medium supplemented with 50 units ml^{-1} penicillin, 50μg ml^{-1} streptomycin and 5% FCS at 15ºC.

Between 1-9 days post treatment the monolayers were washed three times with L-15 medium and the RNA was extracted following the modified TRIzol protocol as described in section 3.2.5. RNA was extracted from macrophage monolayers sampled on a daily basis until 9 days post infection from macrophages groups a and b as described in section. However, RNA was extracted from macrophage groups c – h on 1, 3, 6 and 9 days post infection. In these latter groups, reducing the number of sampling time points lowered the number of macrophages required to perform an experiment. The reduction in sampling

points did not compromise results as the sampling points were carefully selected so as to permit analysis of expression of the chosen targets subsequent to treatment.

5.2.9 Real time q RT-PCR of extracted RNA

The quality and quantity of the extracted RNA were evaluated using a Nanodrop Spectrophotometer. All samples were diluted to 100ng/µl in RNAse/DNAse free water prior to first strand synthesis. Two-step RT-PCR was performed on extracted RNA following the steps outlined in section 4.2.3 and 4.3. All the samples were tested for the presence of Mx, IFN and IPNV. The results of the real time amplification of each target was expressed as a ratio to the internal RT-PCR control ELF-1, using the Pfaffl mathematical equation for relative quantification (Figure 20). Prior to treatments, RNA was extracted from macrophages to identify the normal expression values for each of the targets investigated in the experiment. This time point was termed “time 0” and the experimental data obtained from it was used in the relative quantitation calculation.

5.2.10 Statistical analysis of gene expression in macrophages

Results are expressed as the relative expression ratio between the target of interest and ELF-1 house keeping gene. Data was analysed on the MINITAB software package by a two way ANOVA, and a Tukey’s test was used to perform multiple comparisons to determine the differences between the treatments and time during the course of the experiment. Differences were considered statistically significant when $P<0.05$.

5.2.11 Detection of extracellular IPNV

Prior to extraction of RNA from the macrophage monolayer the culture medium was tested for the presence of extracellular IPNV as described in section 2.2.8.

5.3 Results

5.3.1 IPNV levels in infected macrophages

Levels of IPNV in infected macrophages and uninfected controls were monitored by real time RT-PCR and were calculated relative to the expression of the "housekeeping gene" ELF-1 (Figure 21 and Figure 22). The two way ANOVA results of Figure 23 shows that both treatment, time and a combination of treatment and time had a significant effect on IPNV:ELF-1 expression (P=0.001). The Tukey test revealed that there was a significant difference between IPNV levels immediately after infection (i.e. time 0), and at subsequent time points (day 1 P = < 0.0001, day 3 P = 0.0001, day 5 P = < 0.0001, day 7 P = 0.0002, day 9 P = 0.0006). IPNV levels were elevated at all time points studied, and were greatest at days 1, 3, and 5 post infection Figure 24. These data strongly suggest that IPNV has the ability to replicate in Atlantic salmon head kidney macrophages maintained *in vitro*. No IPNV was detected in uninfected control macrophages.

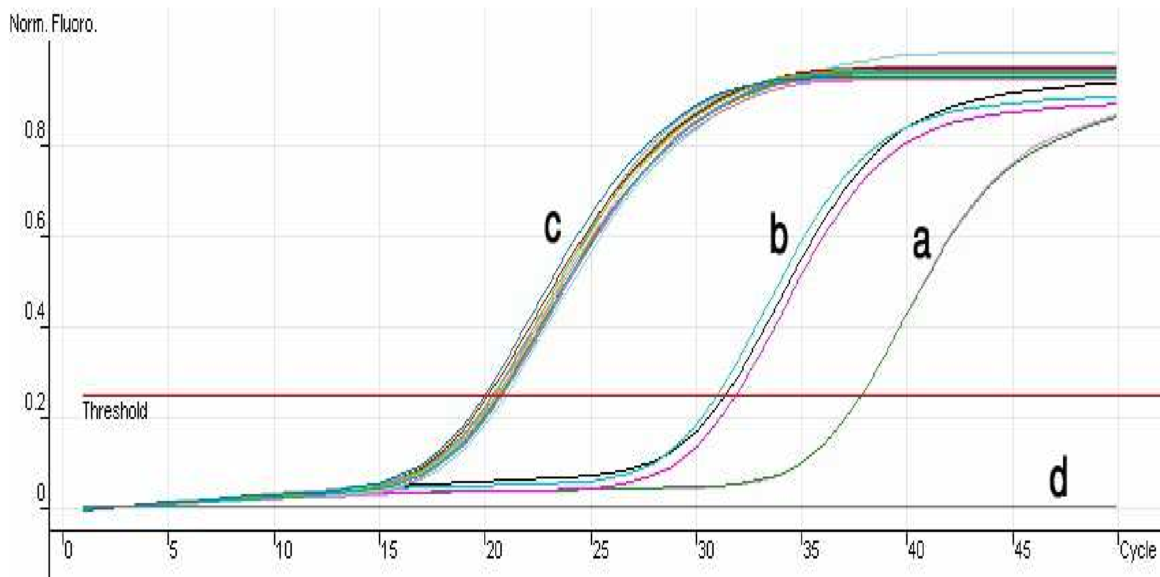


Figure 21: IPNV levels in infected Atlantic salmon macrophages and uninfected controls. The figure shows a RT-PCR amplification plot performed with ELF-1 and IPNV-specific primers and probe of macrophage cDNA. The cDNA samples were taken from IPNV-infected macrophages and controls at 0 hours (a) and 24 hours (b) post infection. IPNV levels were quantified relative to expression of the housekeeping gene ELF-1 (c) using the method described by Pfaffl for relative quantification. The difference in IPNV Ct values between the time 0 and 24h samples (a and b) is clearly evident, whereas the Ct values for ELF-1 from these time points remains relatively constant (c). No template controls and RT- controls were incorporated into amplification runs (d).

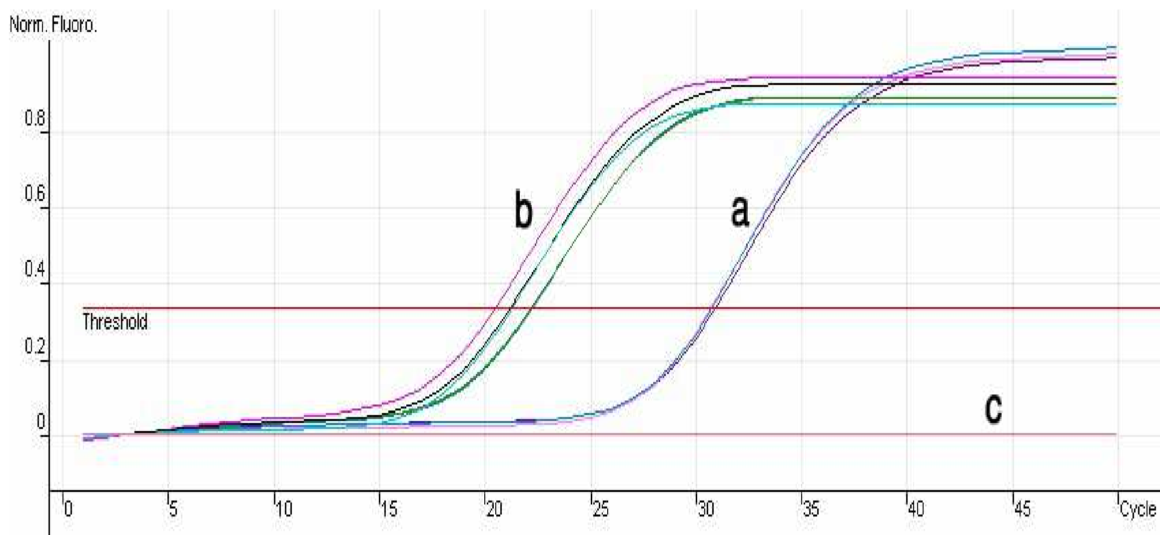


Figure 22: IPNV levels in infected Atlantic salmon macrophages and uninfected controls. The figure shows an RT-PCR amplification plot performed with ELF-1 and IPNV-specific primers and probe of macrophage cDNA. The cDNA samples were taken from IPNV-infected macrophages and controls at 5 days post infection. IPNV levels (a) were quantified relative to expression of the housekeeping gene ELF-1 (b) using the method described by Pfaffl. No template controls and RT- controls were incorporated into amplification runs (d).

Analysis of Variance for Ratio, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	4	346998335	346998335	86749584	20.14	0.001
Treatment	1	246442562	246442562	246442562	57.22	0.001
Time*Treatment	4	346856241	346856241	86714060	20.13	0.001
Error	20	86141676	86141676	4307084		
Total	29	1026438814				

Figure 23: Two way ANOVA results for relative IPNV:ELF-1 expressed in IPNV inoculated Atlantic salmon head kidney macrophages and negative controls over time. Differences were considered statistically significant when P <0.05.

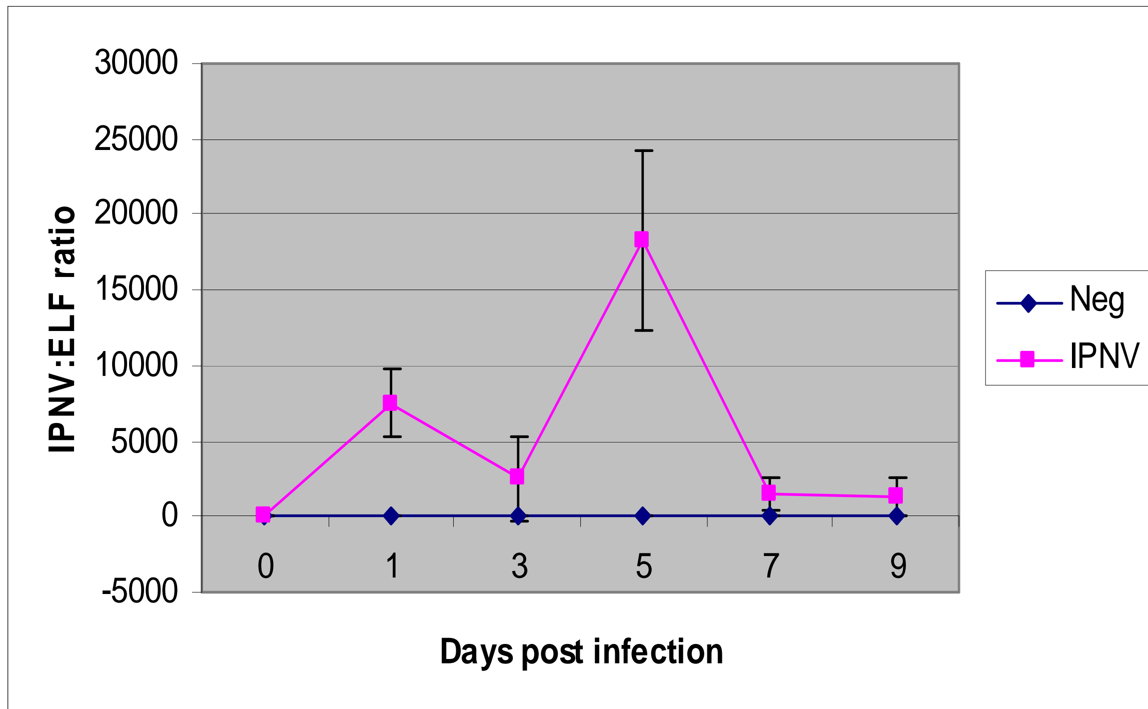


Figure 24: Levels of IPNV in Atlantic salmon head kidney macrophages and uninfected controls detected by qRT-PCR. IPNV levels were studied over a period of 9 days post-infection, and were quantified in relation to expression of the housekeeping gene ELF-1 using method described by Pfaffl.

5.3.2 Preparation of IFN-preparations

IFN for use in macrophage stimulation experiments was produced by harvesting the culture medium of cells stimulated with poly I:C, which is a potent IFN-inducer. The efficacy of IFN preparations was tested with a RTG-1 cell-based assay that utilised a luciferase reporter gene. A triplicate of IFN preparations were tested, these were designated Poly 1, Poly 2, Poly 3 and Poly neg (Figure 25). The one way ANOVA results (Figure 26) shows that there is significant difference in the ability of the IFN supernatants to induce luciferase activity ($P = 0.001$). The tukey test revealed that after 24 hours of stimulation with IFN there was a significant increase in luciferase activity ($P = <0.0001$) in RTG-P1 cells compared to negative controls (RTG-1 cells receiving no Poly I:C treatment or L-15 medium). IFN-preparation “Poly 1” induced expression of the luciferase reporter to the greatest extent; therefore, this preparation was selected for use in macrophage stimulation experiments.

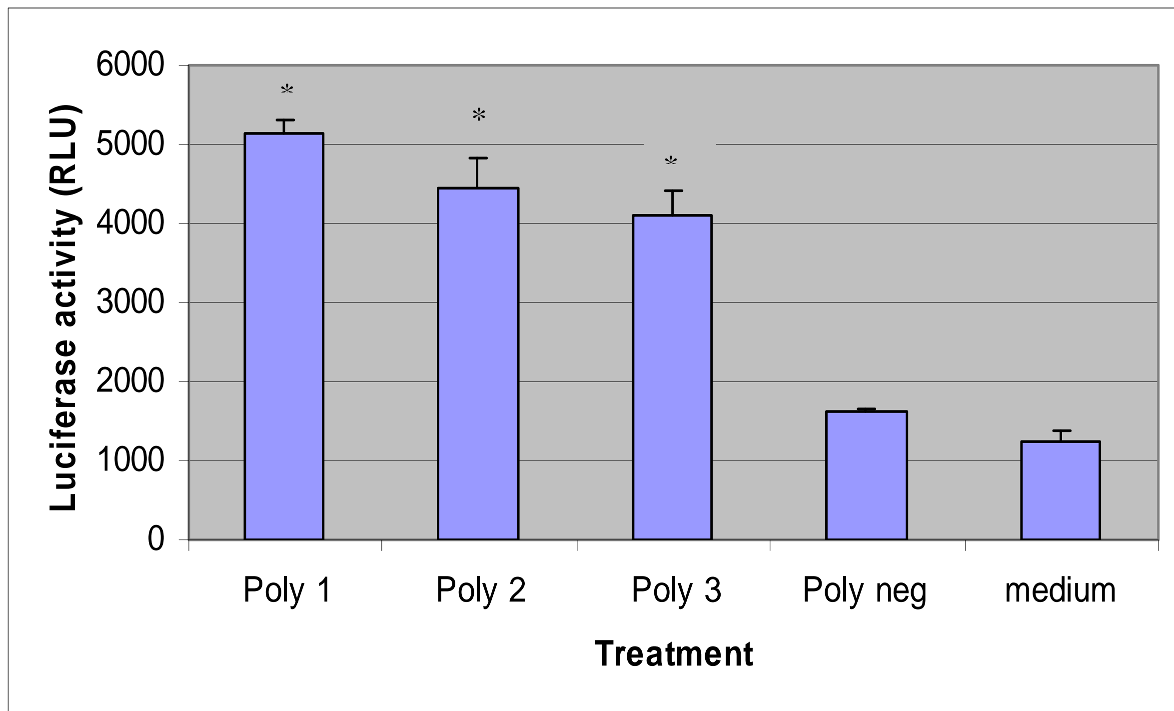


Figure 25: Induction of the Mx-promoter reporter gene activity by IFN containing supernatants in RTG-P1 cells. Histogram bars represent mean luciferase activity expressed in RLU (N = 3) ± SD. * indicates that the RLU value of an IFN preparation was significantly greater than that exhibited by the negative control ($P < 0.05$).

Analysis of Variance for Luciferase activity, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	4	37143087	37143087	9285772	154.99	0.001
Error	10	599132	599132	59913		
Total	14	37742220				

Figure 26: One way ANOVA results for Luciferase activity expressed in RTG-P1 cells stimulated with IFN supernatant compared to negative controls. Differences were considered statistically significant when $P < 0.05$.

5.3.3 Assessment of MAF activity in supernatants

It was not possible to confirm the efficacy of the MAF preparations used in this study. Numerous attempts were performed to test the MAF preparations; however, no positive results were obtained.

5.3.4 Expression of innate immune genes by Atlantic salmon macrophages in response to IFN treatment

According to Collet *et al.,* (2007) the expression of Mx is up-regulated after Poly I:C treatment or incubation with conditioned medium containing an IFN-like activity. Therefore, as Mx is widely regarded as a marker for IFN expression (Robertsen, *et al.,* 1997; Nygaard *et al.,* 2000), incubation of the Atlantic salmon macrophages with the IFN preparations described in section 5.2.1 should induce IFN expression. However, no IFN-expression occurred in any of macrophage groups studied throughout the course of the experiment (Figure 27). The two-way ANOVA report of IFN:ELF-1 expression shows that there is no significant difference in the ability of the treatments (P = 0.948), time (P = 0.130) or a combination of treatment and time (P = 0.089) to induce IFN expression (Figure 28). The tukey test revealed that there was no significant difference in levels of IFN expression between IPNV-infected macrophages and uninfected controls at any sampling point studied (P = 1.0000). However, some experimental groups of macrophages did exhibit elevated expression of Mx expression (Figure 29). This could potentially be a reflection of the time points chosen in this series of experiments. It is

theoretically possible that transient IFN expression could occur in the initial stages of infection. Such expression would not be detected in this experiment because of the timing of sampling points. The two way ANOVA report of Mx:ELF-1 expression shows that there was a significant difference in the ability of the treatments (P = 0.001), time (P = 0.001) and a combination of time and treatment (P = 0.007) to induce Mx expression. The tukey test revealed that uninfected macrophages stimulated with IFN exhibited significantly increased levels of Mx expression as compared to unstimulated controls day 1, 3, 4, 5, 7 P =0.0001; day 2 P = 0.0004; day 8 P = 0.0089; day 9 P = 0.0002) (Figure 29). For both groups of macrophages stimulated with IFN, Mx expression was greatest at 2 days post treatment, and was elevated throughout the course of the experiment. Interestingly, macrophages that were treated with IFN prior to infection with IPNV exhibited levels of Mx expression that closely mirrored those occurring in IFN-treated but uninfected macrophages. There was no significant difference between levels of Mx expression within these two experimental groups at any time point studied (P = 1.0000). Interestingly, Mx expression in IPNV-infected macrophages was not statistically different from that observed in uninfected controls at any of the sampling time points (P >0.9929). This result suggests that IPNV-infected macrophages do not exhibit increased expression of Mx.

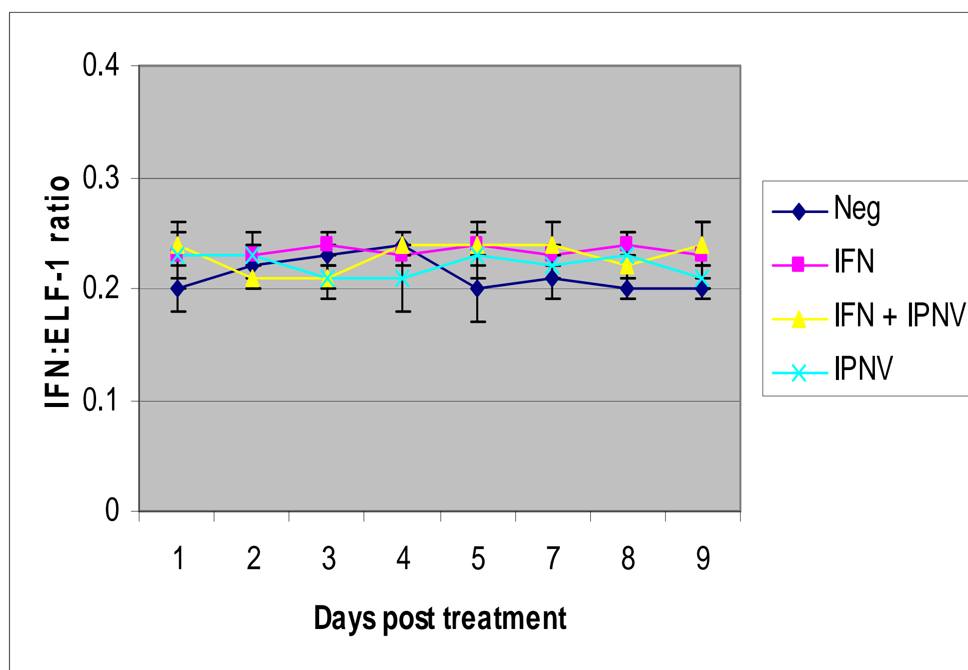


Figure 27: IFN expression in Atlantic salmon head kidney macrophages determined by qRT-PCR. The following experimental groups were studied: (a) IPNV-infected; (b) IFN-stimulated and IPNV-infected; (c) IFN stimulated and (d) untreated macrophages. Expression of IFN was investigated over a period of nine days post-infection and was estimated in relation to expression of the housekeeping gene ELF-1. Data represent mean IFN:ELF-1 ratio (N = 3) ± SD.

Analysis of Variance for IFN:ELF-1 ratio, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	7	0.0020073	0.0020073	0.0002868	1.68	0.130
Treatmen	3	0.0000615	0.0000615	0.0000205	0.12	0.948
Time*Treatmen	21	0.0055969	0.0055969	0.0002665	1.56	0.089
Error	64	0.0109333	0.0109333	0.0001708		
Total	95	0.0185990				

Figure 28: Two way ANOVA results for relative IFN:ELF-1 expression in (a) IPNV-infected; (b) IFN-stimulated and IPNV-infected; (c) IFN stimulated and (d) untreated Atlantic salmon head kidney macrophages over time. Differences were considered statistically significant when $P<0.05$.

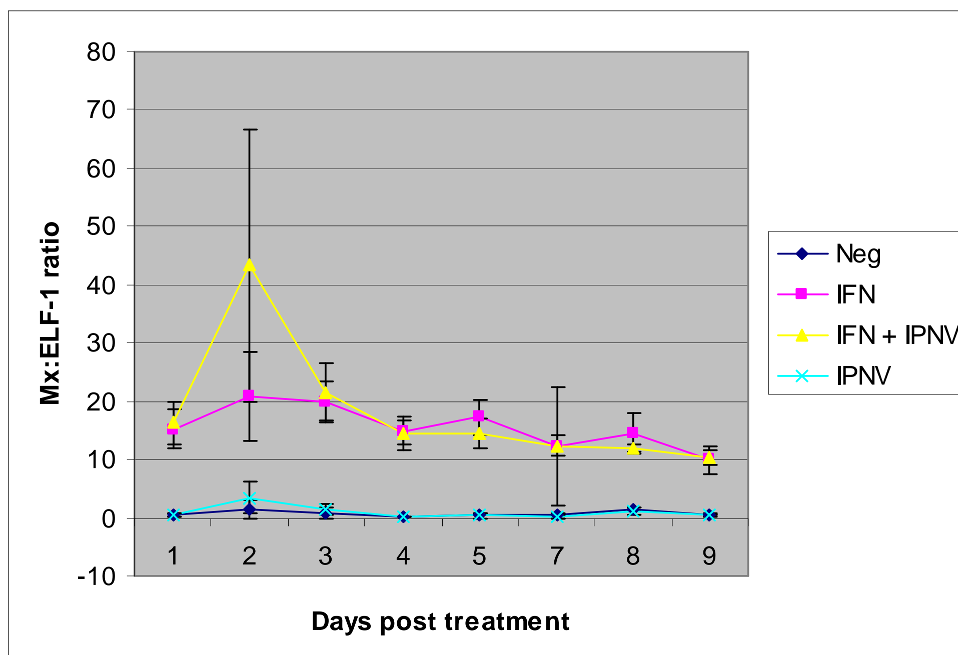


Figure 29: Mx expression in Atlantic salmon head kidney macrophages determined by qRT-PCR. The following experimental groups were studied: (a) IPNV-infected; (b) IFN-stimulated and IPNV-infected; (c) IFN stimulated and (d) untreated macrophages. Expression of Mx was investigated over a period of nine days post-infection and was estimated in relation to expression of the housekeeping gene ELF-1. Data represent mean Mx:ELF-1 ratio (N = 3) ± SD.

Analysis of Variance for Mx:ELF-1 ratio, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	7	31.865	31.865	4.552	10.88	0.001
Treatmen	3	292.005	292.005	97.335	232.69	0.001
Time*Treatmen	21	19.629	19.629	0.935	2.23	0.007
Error	64	26.771	26.771	0.418		
Total	95	370.270				

Figure 30: Two way ANOVA results for relative Mx:ELF-1 expression in (a) IPNV-infected; (b) IFN-stimulated and IPNV-infected; (c) IFN stimulated and (d) untreated Atlantic salmon head kidney macrophages over time. Differences were considered statistically significant when P<0.05.

5.3.5 Expression of innate immune genes by Atlantic salmon macrophages in response to treatment with immunostimulants.

No IFN or Mx expression was detected in macrophages treated with either MAF, glucan, or LPS (Figure 31 and Figure 33). The absence of Mx and IFN expression within immunostimulant treated macrophages may be a consequence of the inability of the these substances to induce a macrophage type I IFN response. The results also suggest that IPNV infection of macrophages may result in suppression of the IFN response. The two-way ANOVA results of Figure 32 shows that neither treatment (P = 0.151), time (P = 0.579) or a combination of treatment and time (P = 0.090) had a significant effect on the expression of IFN. Likewise, the two-way ANOVA results of Figure 34 show that neither treatment (P = 0.381), time (P = 0.914) or a combination of treatment and time (P = 0.232) had a significant effect on Mx expression. The tukey test revealed that there was no significant difference in the IFN expression detected in infected macrophages and in negative controls at any time in the experiment (P = 1.0000). No experimental groups (i.e. experimental groups c-h (section 5.2.8) exhibited an Mx response that was significantly greater than that occurring in negative controls. MAF-containing supernatants, glucan and LPS did not induce Mx expression within macrophages at any time point during the experiment. Interestingly, macrophages failed to produce Mx response in response to IPNV infection, in agreement with results from the IPNV-infected positive control group in the experiment described above (section 5.3.4). IPNV-infected positive control macrophages showed no significant increase in expression of Mx when compared to the negative control macrophages at any of the sampling points studied (P = 1.0000 at days 1, 3 and 6 with P = 0.5844 day 9).

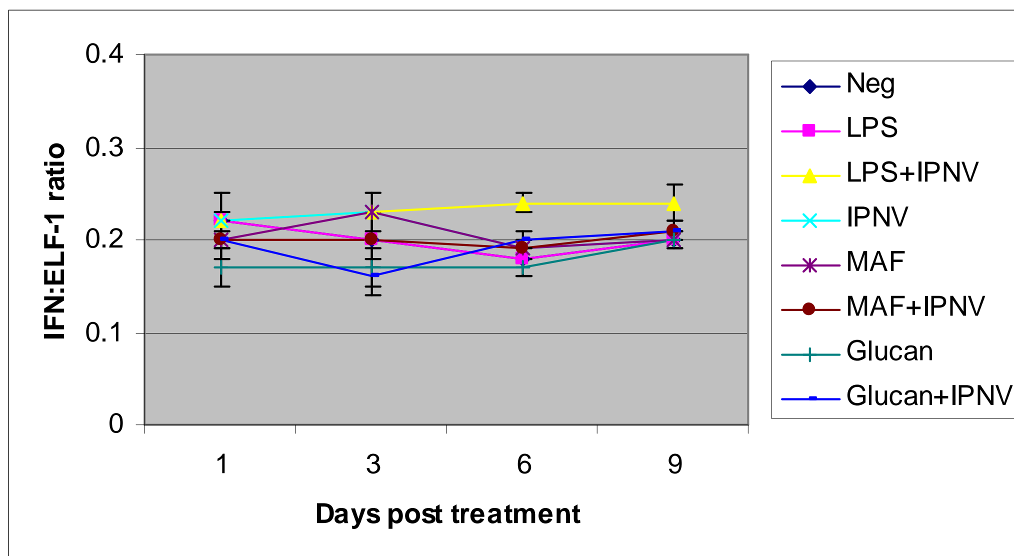


Figure 31: IFN expression in Atlantic salmon head kidney macrophages determined by qRT-PCR. The following experimental groups were studied: (a) IPNV-infected; (b) MAF-stimulated and IPNV-infected; (c) MAF stimulated (d) glucan-stimulated and IPNV-infected; (e) glucan stimulated (f) LPS-stimulated and IPNV-infected; (g) LPS stimulated; (h) Untreated macrophages. Expression of IFN was investigated over a period of nine days post-infection and was estimated in relation to expression of the housekeeping gene ELF-1. Data represent mean IFN:ELF-1 ratio (N = 3) ± SD.

Analysis of Variance for IFN:ELF-1 ratio, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	3	0.0001281	0.0001281	0.0000427	0.66	0.579
Treatmen	7	0.0007240	0.0007240	0.0001034	1.60	0.151
Time*Treatmen	21	0.0021135	0.0021135	0.0001006	1.56	0.090
Error	64	0.0041333	0.0041333	0.0000646		
Total	95	0.0070990				

Figure 32: Two way ANOVA results for relative IFN:ELF-1 expression in (a) IPNV-infected; (b) MAF-stimulated and IPNV-infected; (c) MAF stimulated (d) glucan-stimulated and IPNV-infected; (e) glucan stimulated (f) LPS-stimulated and IPNV-infected; (g) LPS stimulated; (h) Untreated Atlantic salmon head kidney macrophages over time. Differences were considered statistically significant when P<0.05.

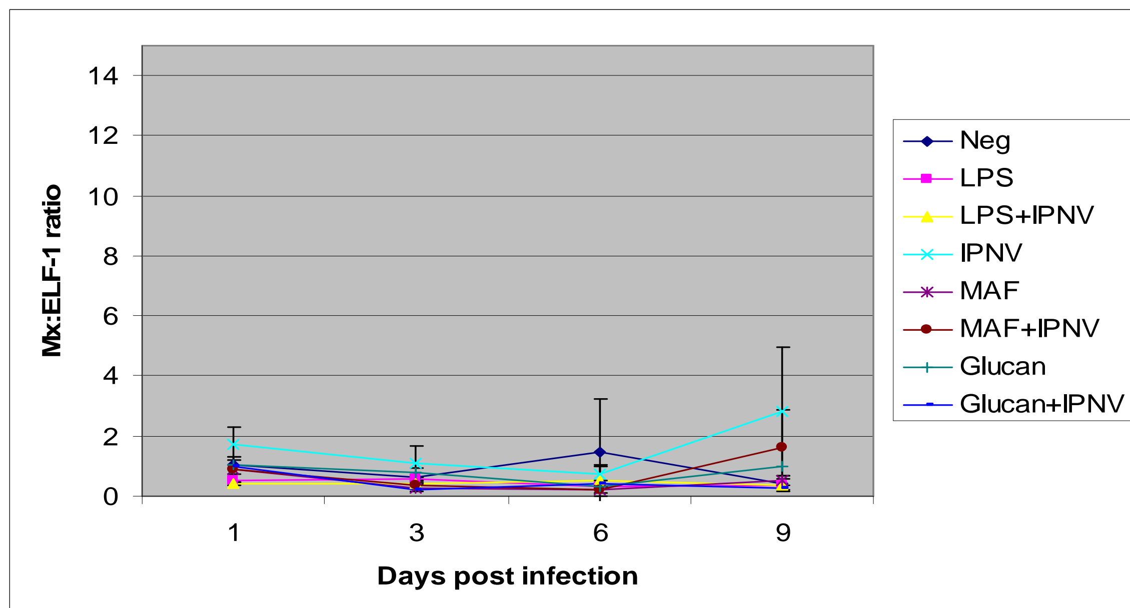


Figure 33: Mx expression in Atlantic salmon head kidney macrophages determined by qRT-PCR. The following experimental groups were studied: (a) IPNV-infected; (b) MAF-stimulated and IPNV-infected; (c) MAF stimulated (d) glucan-stimulated and IPNV-infected; (e) glucan stimulated (f) LPS-stimulated and IPNV-infected; (g) LPS stimulated; (h) Untreated macrophages. Expression of Mx was investigated over a period of nine days post-infection and was estimated in relation to expression of the housekeeping gene ELF-1. Data represent mean Mx:ELF-1 ratio (N = 3) ± SD.

Analysis of Variance for Mx:ELF-1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	3	0.0000365	0.0000365	0.0000122	0.17	0.914
Treatmen	7	0.0005323	0.0005323	0.0000760	1.09	0.381
Time*Treatmen	21	0.0018552	0.0018552	0.0000883	1.27	0.232
Error	64	0.0044667	0.0044667	0.0000698		

Figure 34: Two way ANOVA results for relative Mx:ELF-1 expression in (a) IPNV-infected; (b) MAF-stimulated and IPNV-infected; (c) MAF stimulated (d) glucan-stimulated and IPNV-infected; (e) glucan stimulated (f) LPS-stimulated and IPNV-infected; (g) LPS stimulated; (h) Untreated Atlantic salmon head kidney macrophages over time. Differences were considered statistically significant when $P<0.05$.

5.3.6 IPNV levels in IFN-treated macrophages

Levels of IPNV in infected macrophages treated with IFN and untreated controls (i.e. experimental groups b and i, section 5.2.8) were monitored by real-time RT-PCR. IPNV was detected in both experimental groups of macrophages that were infected with this virus (Figure 35). These included macrophages that had been exposed to IFN prior to infection indicating that IFN does not protect macrophages from infection with IPNV. The two-way ANOVA results (Figure 36) show that treatment ($P = 0.001$), time ($P = 0.002$) and a combination of time and treatment ($P = 0.001$) had a significant effect on IPNV expression. The tukey test revealed that the negative control macrophages exhibited statistically different IPNV:ELF-1 expression compared to those treated with IFN + IPNV ($P = <0.0001$) and IPNV ($P = <0.0001$ on all days apart from day 2; $P = 0.9929$) throughout the duration of the experiment. IPNV levels were elevated at 1 day post-inoculation, and peaked at 5 days in positive control infected macrophages. However, there was no significant difference between the IPNV:ELF-1 ratios on day 1 and 5 post infection ($P = 1.0000$) or day 4 and 5 post infection ($P = 0.9222$). Infected macrophages stimulated with IFN exhibited peak virus levels at 3 days post-inoculation. However there was no significant difference between day 1 and 3 ($P =1.0000$) or day 2 and 3 ($P = 1.0000$). Therefore, as an increase in the mean IPNV:ELF-1 data was observed (Figure 35) it is probable that the virus replicated in the macrophages following infection at a low level. Virus levels tended to decrease at the later time points; therefore, it is possible that intracellular virus may have been broken down by the macrophages. The absence of infectious IPNV in the growth media of infected macrophages as assessed by virus isolation in CHSE-214 cells lends support to this conclusion. There was a

significant difference between the IPNV:ELF-1 ratio occurring on day 1 and that occurring on day 9 for IFN-stimulated and IPNV-infected macrophage groups ($P = 0.0011$). However there was no significant difference in IPNV levels on day 1 ($P = 0.9503$) day 5 ($P = 0.1170$) and day 9. This suggests that IFN treatment promotes a reduction in IPNV levels in infected macrophages, which could limit the ability of IPNV to persist in these cells. No IPNV was detected in negative control groups (uninfected-macrophages, and IFN stimulated uninfected macrophages).

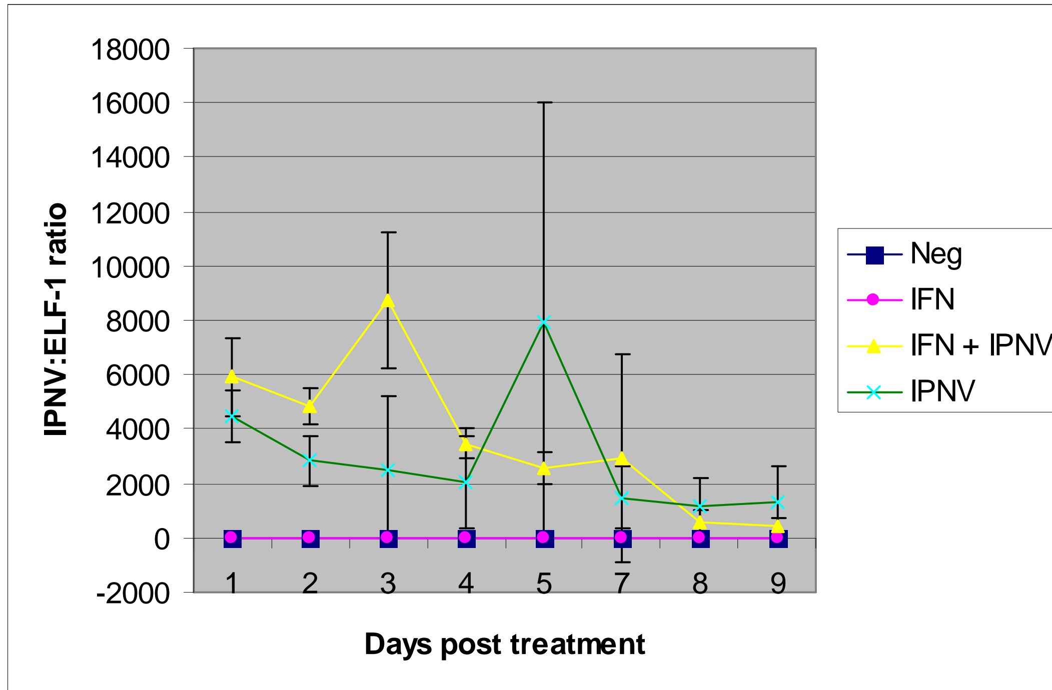


Figure 35: IPNV expression in Atlantic salmon head kidney macrophages determined by qRT-PCR. The following experimental groups were studied: (a) IPNV-infected; (b) IFN-stimulated and IPNV-infected; (c) IFN stimulated and (d) untreated macrophages. Expression of IPNV was investigated over a period of nine days post-infection and was estimated in relation to expression of the housekeeping gene ELF-1. Data represent mean IPNV:ELF-1 ratio (N = 3) ± SD.

Analysis of Variance for IPNV:ELF-1, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	7	11.594	11.594	1.656	3.70	0.002
Treatmen	3	1598.458	1598.458	532.819	1189.14	0.001
Time*Treatmen	21	32.432	32.432	1.544	3.45	0.001
Error	64	28.676	28.676	0.448		
Total	95	1671.160				

Figure 36: Two way ANOVA results for relative IPNV:ELF-1 expression in (a) IPNV-infected; (b) IFN-stimulated and IPNV-infected; (c) IFN stimulated and (d) untreated Atlantic salmon head kidney macrophages over time. Differences were considered statistically significant when $P<0.05$.

5.3.7 IPNV levels in immunostimulant-treated macrophages.

Treatment of macrophages (experimental groups c-h section 5.2.8) with immunostimulants did not prevent infection with IPNV, since infected macrophages exhibited raised IPNV:ELF-1 ratios compared to uninfected cells (Figure 37). The two-way ANOVA results (Figure 38) shows that treatment (P = 0.001), time (P = 0.001) and a combination of treatment and time (P = 0.012) had a significant effect on IPNV expression. The tukey test revealed that a significant difference in IPNV:ELF-1 ratios was evident between the negative control macrophages and those exposed to LPS and IPNV, IPNV, MAF and IPNV, and glucan and IPNV treatments throughout the duration of the experiment (P = 0.0001). As expected macrophages treated only with immunostimulants and the negative control treatment group showed the lowest IPNV:ELF-1 ratio of all experimental groups. Macrophages receiving only glucan, LPS or MAF were all shown to have significantly different IPNV:ELF-1 ratios (P = 0.0001) compared to those receiving glucan + IPNV, LPS + IPNV or MAF + IPNV for all of the sampling points. The highest IPNV:ELF-1 ratios were observed in macrophages treated with MAF and glucan prior to exposure to IPNV. Macrophages stimulated with MAF prior to infection with IPNV were found to have significantly different IPNV:ELF-1 ratios compared to IPNV infected positive control macrophages at 6 days (P = 0.0001) and 9 days post infection (P = 0.0015). Similarly, macrophages stimulated with glucan prior to infection were also found to have significantly different IPNV:ELF-1 ratios compared to IPNV infected positive control macrophages on day 6 (P =0.0071) and 9 days post treatment (P = 0.0091). Macrophages stimulated with MAF and glucan prior to infection exhibited similar levels of IPNV:ELF-1 ratios over time, with increases evident

from day 1 that peaked at day 6, however these increases were not statistically significant for either MAF (P =0.2069) or glucan (P =0.4628). IPNV:ELF-1 ratios subsequently declined between days 6 and day 9. Whilst Figure 37 showed an increase in IPNV:ELF-1 ratios during the experiment, the increase in IPNV:ELF1 ratios between day 1 and 9 post infection was not proven significantly different in the macrophages stimulated with MAF (P = 0.6733), glucan (P = 0.7128) or LPS (P = 0.1934) prior to infection. In the positive control group there was also no significant difference between the day 1 and 9 IPNV:ELF-1 ratios (P = 0.9999). As observed in the IPNV infected macrophages in the IFN experiment (section 5.3.6) whilst there appears to be an increase in the IPNV levels (Figure 35), this did not attain statistical significance. Therefore, it is likely that the virus replicated at low levels in the macrophages following infection. This suggests that, as there was no significant reduction in the viral levels during the experiment MAF, glucan or LPS are unlikely to represent effective treatments for IPN. However, it is possible that these substances could influence immune function in cell types other than macrophages. No IPNV was detected in negative control groups (uninfected macrophages, and MAF, glucan and LPS stimulated uninfected macrophages).

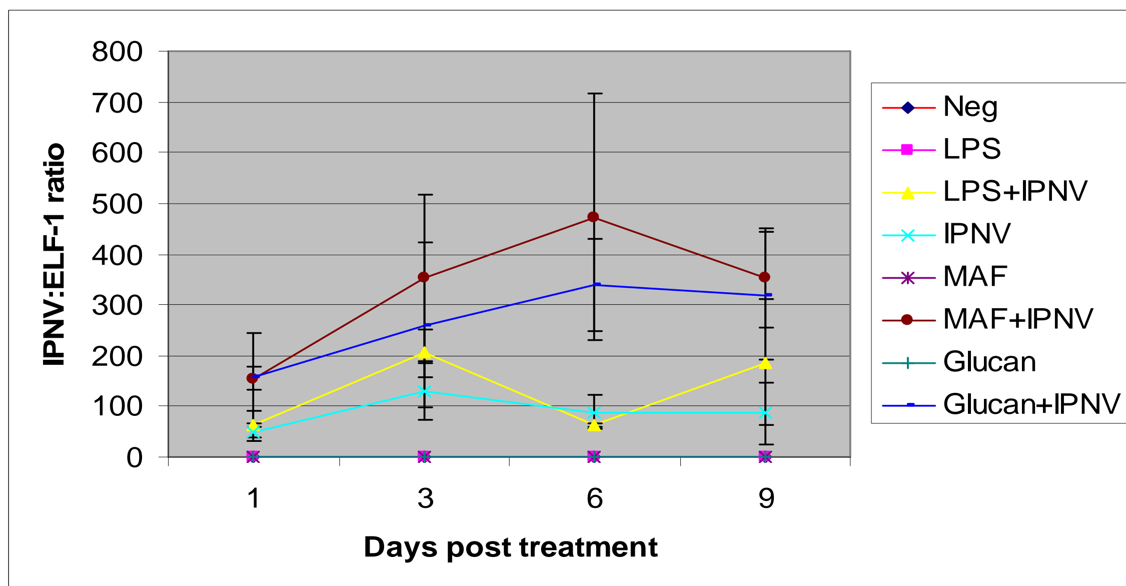


Figure 37: IPNV expression in Atlantic salmon head kidney macrophages determined by qRT-PCR. The following experimental groups were studied: (a) IPNV-infected; (b) MAF-stimulated and IPNV-infected; (c) MAF stimulated (d) glucan-stimulated and IPNV-infected; (e) glucan stimulated (f) LPS-stimulated and IPNV-infected; (g) LPS stimulated; (h) untreated macrophages. Expression of IPNV was investigated over a period of nine days post-infection and was estimated in relation to expression of the housekeeping gene ELF-1. Data represent mean IPNV:ELF-1 ratio (N = 3) ± SD.

Analysis of Variance for IPNV:ELF-1 ratio, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	3	12.785	12.785	4.262	31.12	0.001
Treatment	7	706.160	706.160	100.880	736.69	0.001
Time*Treatment	21	6.080	6.080	0.290	2.11	0.012
Error	64	8.764	8.764	0.137		
Total	95	733.789				

Figure 38: Two way ANOVA results for relative IPNV:ELF-1 expression in (a) IPNV-infected; (b) MAF-stimulated and IPNV-infected; (c) MAF stimulated (d) glucan-stimulated and IPNV-infected; (e) glucan stimulated (f) LPS-stimulated and IPNV-infected; (g) LPS stimulated; (h) untreated Atlantic salmon head kidney macrophages over time. Differences were considered statistically significant when P<0.05.

5.3.8 Determination of IPNV levels in the culture media of infected macrophages

No CPE was observed in CHSE-214 monolayers inoculated with culture media from any experimental group of IPNV-infected macrophages. This indicates that IPNV-infected macrophages do not release virus into the macrophage culture medium during the course of the experiments. In response to IPNV infection, it has been observed in both experiments that the IPNV-infected macrophages do not express Mx (sections 5.3.4 and 5.3.5). This observation, combined with the results of the present experiment suggest that IPNV may have the ability to suppress the type I IFN response in macrophages, which in turn may facilitate the development of a carrier state that occurs in IPN of Atlantic salmon.

5.4 Discussion

5.4.1 IPNV levels in infected macrophages *in vitro*

IPNV:ELF-1 ratios increased between time 0 and 1 day post infection (Figure 24, $P = <0.0001$) demonstrating that it is possible to infect Atlantic salmon head kidney macrophages with IPNV *in vitro*. In the context of the aims of this thesis, this validation is essential. Without confirmation that it is possible to successfully infect macrophages *in vitro* would not be possible to conclude that IPNV levels are associated with replicating virus as opposed to virus which has adhered to cells and then decayed during the course of experiments. Furthermore, without this data, it would not be possible to draw conclusions on the ability of immunostimulants to mitigate infection or to aid

macrophages in clearing virus. Figure 24 indicates that IPNV levels in macrophages are elevated in comparison to those occurring immediately after infection at all time points studied. The data also suggest that peak levels of IPNV replication occur in the early stages of infection, and that replication declines at the later time points studied. The persistence of low levels of virus in macrophages *in vitro* for up to 9 days, as noted in this study, is in agreement with the findings of Yu *et al.,* (1982), Johansen and Sommer (1995), Novoa *et al.,* (1996), Munro *et al.,* (2006) and Collet *et al.,* (2007) However only Yu *et al.,* (1982) and Johansen and Sommer (1995) have suggested that IPNV can replicate within macrophages *in vitro*. The results of the present study therefore agree with other studies that suggest there is a potential role for head kidney macrophages in maintaining an IPNV carrier state in Atlantic salmon. The results of this experiment indicate that IPNV replicates within macrophages *in vitro*. On this basis, it was possible to pursue the subsequent objectives of this thesis. These are described in the ensuing sections of this chapter, and in chapter 6.

5.4.2 Effect of IPNV infection on the innate immune response of macrophages *in vitro*

One objective of this chapter was to investigate the effect of IPNV infection on macrophage expression of Mx, an important component of the anti-viral innate immune response. Stimulation of macrophages with IFN was studied to determine the potential of this IFN as a means of preventing IPNV infection or persistence, either directly or through the use of IFN-inducing immunostimulants. The results indicate that Atlantic salmon macrophages maintained *in vitro* do exhibit an Mx response when stimulated with

IFN. The efficacy of the IFN preparations used in this study was confirmed with reporter gene assays performed in RTG-P1 cells, which are a rainbow trout fibroblastic cell line permanently transfected with the luciferase gene under the control of Mx promoter (Figure 25). On exposure to IFN or IFN-inducing agents, the RTG-P1 cells express luciferase, (Johansen *et al*., 2004). Each of the three IFN preparations (Poly 1, 2, 3) produced in this study exhibited IFN-inducing abilities. The first experiment in this chapter was aimed at providing an answer to the following questions summarised in the subsequent sections (5.4.3 to 5.4.5.3).

5.4.3 Does IPNV induce an Mx response, and how does this compare to that induced by IFN?

Little is known about the molecular and immunological mechanisms involved in the establishment of an IPNV carrier state (Santi *et al.,* 2005). IPNV-infected macrophages did not induce an Mx response at any time point studied (Figure 29). Whilst an Mx response was induced within macrophages stimulated with IFN prior to IPNV infection, this was not found to be significantly different from uninfected controls at any sampling time point studied. This suggests that IPNV has evolved in such a way so as not to induce an Mx response. Larsen *et al.,* (2004) transfected CHSE-214 cells with the Atlantic salmon Mx1 protein (ASMx1) and demonstrated the antiviral effect of these cells towards IPNV. This finding indicates that Mx is capable of inhibiting IPNV replication. However, the results of the present study suggest that IPNV may suppress the immune response within head kidney macrophages, facilitating persistence and the development of a carrier state. The type I IFN system has a crucial role in the first line of defence against virus

infections (Johansen *et al.,* 2004). The IFNs provide vertebrates with a first line of defence against viral infection. These cytokines are produced by virus-infected cells and stimulate the production of IFN-induced proteins in neighbouring, uninfected cells. These IFN-induced proteins in turn, confer an anti-viral state upon uninfected cells (Trobridge *et al.,* 1997). According to Levy and Garcia-Sastre (2001), the IFN system is one of the earliest defence mechanisms against virus infections acquired during evolution by higher eukaryotes. Not surprisingly, millions of years of co-evolution between hosts and their pathogens have resulted in the acquisition of mechanisms by most viruses to inhibit, at least to some extent, the host IFN system. In this study, no IFN expression was detected by real time RT-PCR in any of macrophage groups studied (Figure 27). There was no significant difference in IFN expression exhibited by treated macrophages and untreated controls throughout the experimental study period. As it was possible to detect the presence of Mx in macrophages exposed to IFN, it was assumed IFN expression occurred early in the course of infection at a time not covered by the initial sampling points. IFN's are unstable molecules that are rapidly degraded. Mx proteins however, which are strictly induced by type I IFNs, can persist for weeks after virus infection (Bergan and Robertsen, 2004). Consequently, Mx has been regarded as a good molecular marker for type I IFN (Robertsen, *et al.,* 1997; Nygaard *et al.,* 2000). As a result, where Mx was detected within macrophages, it can be assumed that IFN induction occurred. However, this assumption cannot be made with respect to IPNV-infected macrophages, as it was not possible to detect the presence of Mx. Collet *et al.,* (2007) reported the detection of IFN in the supernatant of Atlantic salmon head kidney macrophages following *in vitro* infection with IPNV, however in a parallel experiment IPNV suppressed Mx expression in RTG-

P1 cells. Unfortunately Collet *et al.,* (2007) did not investigate expression of Mx in macrophages, confounding attempts to make comparisons with the present study. Opinion is divided as to whether IPNV induces IFN in fish cells. It has been shown that head kidney leucocytes from rainbow trout expressed Mx mRNA when infected *in vitro* with IPNV (Boudinot *et al.,* 1999). However, IPNV infection *in vitro* has been shown not to induce an Mx response in the TO-cell line (Jensen and Robertsen, 2002) which originates from Atlantic salmon head kidney (Wergeland and Johansen, 2001). Jensen and Robertsen (2000) reported that Mx was strongly induced *in vivo* by IPNV in Atlantic halibut. It is possible that the ability of IPNV to induce Mx may be dependent upon cell type (Jensen and Robertsen, 2002; Bergan and Robersen, 2004; Collet *et al.,* 2007) or due to temperature effects (Bergan and Robersen, 2004). For a virus to persist within its host, it must actively curtail or evade the antiviral immune response (Oldstone, 2006), the same author suggests that there are certain foundations upon which the understanding of persistent infection rests. One of these is that the host's immune response fails to form or fails to purge virus from the infected host. Thus, viral persistence is synonymous with evasion of the host's immunologic surveillance system. Another foundation that Oldstone (2006) describes is that viruses can acquire unique component(s) or strategies of replication. That is viruses can regulate expression of both their own genes and host genes to achieve residence in a non-lytic state within the cells they infect. From the results of this experiment, it appears that both these statements made by Oldstone (2006) apply to the observations described in the results of this experiment and propose how IPNV is able to persist within Atlantic salmon head kidney macrophages.

5.4.4 Does IFN reduce IPNV levels in Atlantic salmon macrophages?

Little is known about the mechanisms involved in viral persistence and viral carrier states in fish and how viruses evade host defences (Ellis, 2001). In the present study, treatment of macrophages with IFN was used to activate antiviral host responses prior to infection with IPNV. Through a comparison of the levels of IPNV:ELF-1 ratios in IFN stimulated and unstimulated macrophages it was possible to investigate the effect of the antiviral state on IPNV replication. The results indicate that macrophages become infected with IPNV despite the presence of an Mx response. Figure 35 shows that there was an increase in the mean IPNV levels in both the IFN-stimulated macrophages and positive controls, suggesting that the virus may have replicated after infection at a low level in the macrophages. However, these differences did not attain statistical significance.

Johansen and Sommer (1995) reported increases in both intracellular and extracellular IPNV levels in adherent head kidney leucocytes, originating from IPNV carrier Atlantic salmon, during 7 days in culture and demonstrated that IPNV multiplied in these cells. However, the results of other studies do not support the idea that IPNV replicates in head kidney macrophages. Munro *et al.,* (2006) demonstrated that IPNV persisted within Atlantic salmon head kidney macrophages infected *in vitro* over a period of 7 days without replicating. Collet *et al.,* (2007) similarly demonstrated that *in vitro* infection of Atlantic salmon head kidney macrophages resulted in the persistence of the virus for nine days within the cells without replicating. Nova, *et al.,* (1996b) and Yu *et al.,* (1982) both suggested that although adherent rainbow trout macrophages harbour non-replicating IPNV, the virus replicates in other cell types. Nova *et al.,* (1996) demonstrated that IPNV did not replicate in rainbow trout macrophages cultured *in vitro*,

but a limited increase in viral titre was observed when total leucocytes were infected with the culture. Whilst the results of this thesis would appear to suggest that it is possible for IPNV to replicate in Atlantic salmon head kidney macrophages *in vitro*, they do not agree with the majority of published studies. Unfortunately, it was only possible to investigate virus levels in macrophages using relative quantification, however a more accurate assessment of the ability of IPNV to replicate within macrophages *in vitro* could be achieved using absolute quantification. Financial and logistical constraints prevented this approach in the present study.

In IFN treated macrophages infected with IPNV, the IPNV:ELF-1 ratio was significantly different between day 1 and day 9 ($P = 0.0011$), however in unstimulated IPNV-infected macrophages the IPNV:ELF-1 ratio was not statistically different at these times ($P = 0.1932$). This result suggests that IFN stimulation reduces IPNV levels in macrophages after infection. This corresponds with the results of section 5.4.3, which demonstrate that macrophages stimulated with IFN prior to infection with IPNV exhibit significantly greater levels of Mx expression than unstimulated IPNV-infected macrophages. This suggests that IPNV have evolved the capability to suppress the type I IFN system in Atlantic salmon macrophages. Macrophages stimulated with type I IFN, should express IFN-inducible antiviral mechanisms when subsequently infected with IPNV. Thus, those antiviral responses which would under normal circumstances be suppressed by IPNV should be active within the macrophages, explaining the difference in virus levels in these two experimental groups of macrophages. Although these results suggest that IFN may have potential as a means of reducing IPNV levels in Atlantic salmon, the differences in the mean IPNV:ELF-1 ratios were shown to decline between

day 1 (4486.8) and 9 (1320.7) in the positive control IPNV infected macrophages even though they were not exposed to IFN. Whilst this decline was not statistically significant, it could be argued that a decline in the IPNV:ELF-1 ratio still occurred with the IPNV-infected control group. As no Mx was detected in this experimental group, this therefore raises questions over the exclusive role of Mx in reducing IPNV levels in infected macrophages. The observed reduction in IPNV levels within infected macrophages is unlikely to be due to shedding of the virus. No IPNV was detected in the macrophage culture medium as judged by virus isolation in CHSE-214 cells. Washing of macrophage cultures and replacement of culture medium took place every second day as recommended by Secombes (1990) and it is theoretically possible that this will have led to the removal of any IPNV shed into the media. However, daily sampling of the culture medium took place prior to any washing and renewal of the culture medium to ensure that extracellular virus would not be lost. Munro *et al.,* (2006) studied the level of infection by IPNV of kidney macrophages from asymptomatic carrier Atlantic salmon post-smolts. The macrophages were cultured for up to 7 days with or without renewal of medium on day 3. Their results showed that the removal of the medium on day 3 had a significant effect on IPNV persistence within macrophages. In cultures where the medium was not removed, on day 7, IPNV was detected in macrophage lysates and the supernatants were also found to be IPNV positive. However where the medium was renewed IPNV was not detected. Munro *et al*., (2006) concluded that IPNV might persist within a macrophage population for long periods by circulating amongst cells.

IFN and Mx were the only immune markers investigated in the present study due to financial and logistical restrictions. It is possible that other components of the immune

response may have been responsible for the decline in the IPNV detected in infected macrophages. A cascade of cytokines is released in macrophages as part of the innate immune response (Bird *et al.,* 2005). However, very little is known about the role of cytokines in fish antiviral responses (Tafalla *et al.,* 2005). In the last few years, many cytokine and other immune related genes have been identified in different teleost species, thus facilitating their study at a molecular level, these include tumour necrosis factor-α (TNF-α); (Zou *et al.,* 2002), interleukin- 1ß (IL-1ß); (Bird *et al.,* 2005), interleukin-6 (IL-6); (Bird *et al.,* 2005), interleukin-8 (IL-8); (Laing *et al.,* 2004). TNF-α is primarily a product of monocytes and macrophages that have been activated by foreign substances including viruses, and is the principal mediator of the host inflammatory response (Secombes, 1994). According to McBeath *et al.,* (2007) TNF-α is often associated with type I IFN production following a viral infection. Yoshiura *et al.,* (2003) reported that IL-12 in fish may be involved in antiviral defence. Tafalla *et al.,* (2005) observed that rainbow trout infected with viral haemorrhagic septicaemia virus (VHSV) showed increased IL-1ß transcription. Semi-quantitative RT-PCR showed the virus induced an increased transcription of IL-1β in the spleen and to a lesser extent in the head kidney and liver at early times post-infection. However, it is unlikely that any of these cytokines played a role in the decrease of the IPNV levels. McBeath *et al.,* (2007) measured several aspects of the Atlantic salmon immune response following experimental infection with IPNV. They demonstrated that the IPNV failed to induce the expression of TNF-α and IL-1ß. Interestingly, McBeath *et al.,* (2007) reported that Type II IFN was greatly upregulated in IPNV infected fish. The salmon type II IFN gene has been shown to function in fish in a similar manner to mammals (Zou *et al.,* 2005). For many years type

II IFN was thought to be only expressed by T cells, however in recent years it has been demonstrated that other cell types, originally thought not to be producers of type II IFN, are in fact capable of IFN expression. Type II IFN production has been reported in human macrophages (Gessani and Belardelli, 1998; Frucht *et al.,* 2001; Ellermann-Eriksen, 2005). The results of McBeath *et al.,* (2007) could not verify if the peak type II IFN expression in response to IPNV was the result of activation of antigen-specific cytotoxic CD8+ T-cells, macrophages or NK cells. Unfortunately, due to financial constraints it was not possible to investigate expression of other immune markers, such as those involved in the type II IFN pathway.

No CPE was evident in these macrophages during the course of the experiment. The strain of IPNV used in this study induced CPE within 2 days in CHSE-214 cells, thus this strain of IPNV is capable of producing extensive CPE. The results of the present study are in agreement with those of Estapa and Coll (1991), Johansen and Sommer (1995), and Collet *et al.,* (2007) who showed that in both rainbow trout and Atlantic salmon, *in vitro* infection of macrophages with IPNV did not lead to a CPE, suggesting that the level of virus was reduced by a non-cytolytic process.

5.4.5 Do immunostimulants induce IFN and Mx responses in macrophages and do immunostimulant-treated macrophages clear IPNV?

The remainder of this chapter is focused on the potential of three immunostimulant; (MAF, glucan and LPS), to protect Atlantic salmon head kidney macrophages from infection with IPNV. Bricknell and Dalmo (2005) have stated that the theoretical benefit of immunostimulants is considerable. They have the potential to

elevate the innate immune defence mechanisms of fish prior to the exposure to a pathogen, or improve survival following exposure to a specific pathogen when treated with an immunostimulant. The majority of publications in the scientific literature on LPS and glucan as immunostimulants are concerned with bacterial diseases, dealing with their ability to stimulate the respiratory burst of macrophages and lysozyme activity (Solem, *et al.,* 1995; Neumann, *et al.,* 1995; Jørgensen and Robertsen, 1995; Robertsen, 1999; Paulsen, *et al.,* 2003; Bridle *et al.,* 2005). Studies on the protection against viral infection offered by immunostimulants are scarce, and little is known about their IFN and Mx inducing properties in fish (Salinas, *et al.,* 2004).

5.4.5.1 Glucan

ß-Glucan potentiates and modulates the immune response primarily through its effects on macrophage and reticulo-endothelial cells and is generally recognized as safe without toxicity or side effects (Kumari and Sahoo, 2006). According to Bricknell and Dalmo (2005), one of the earliest applications of immunostimulants in aquaculture was the use of glucan in salmon diets. These diets were considered to be effective in managing disease outbreaks after stressful events such as grading. In the present study, the glucan used was Laminarin, a ß (1,6)-branched ß (1,3)-D-glucan, which is a major component in sublittoral brown algae and occurs principally in the Laminariae (Peat *et al.,* 1958). Laminaran has the potential to enhance the non-specific defence against infectious diseases, administered either perorally as a feed additive, or intraperitoneally by injection (Dalmo *et al.,* 1996).

The results of this experiment (section 5.3.5) show that glucan was unable to induce IFN (Figure 31) or Mx (Figure 33) expression in Atlantic salmon head kidney

macrophages. There was no significant difference between the Mx:ELF-1 ratios observed in glucan-stimulated macrophages, glucan stimulated IPNV-infected macrophages and negative controls This result is in agreement with other published studies. Robertsen *et al.,* (1997) compared the ability of glucan to induce an Mx response in trout and salmon. Glucan was not able to induce Mx expression in either species by injection. Salinas *et al.,* (2004) reported the same observations when they conducted experiments on Atlantic salmon parr and concluded that neither glucan or yeast are capable of stimulating the type I IFN pathway in salmon parr. Kumari and Sahoo (2006) report that glucan incorporated into Asian catfish (*Clarias batrachus* L.) feed was able to successfully activate non-specific immune functions such as lysozyme, and superoxide production, which led to protection against septicaemia caused by the motile aeromonad *Aeromonas hydrophila*. Thus, glucan has been shown to increase the resistance of fish to bacterial diseases. (Robertsen *et al*., 1990; Chen and Ainsworth, 1992; Guselle *et al.,* 2006; Kumari and Sahoo, 2006). Engstad and Robertsen (1993) report that Atlantic salmon macrophages express a specific receptor for glucan, which supports a role for macrophages in glucan-induced antibacterial responses in fish. This finding could explain why glucan has been successfully used to control bacterial diseases in fish and can also possibly explain the inability of glucan to induce an Mx response. It is possible that the putative glucan receptor is not coupled to the IFN or Mx pathways.

The inability of glucan to induce an antiviral response in Atlantic salmon head kidney macrophages was in agreement with the observed IPNV levels in infected macrophages that were stimulated with glucan prior to infection. Between day 1 and 6 there was a marked increase in intracellular IPNV:ELF-1 ratios (156.17 to 339.85 -

Figure 37). IPNV levels in glucan-treated macrophages were higher than those occurring in untreated controls. Initially this increase would appear to suggest that IPNV replicated to high levels until 6 days post infection. However in the same time period the IPNV:ELF-1 ratio in the IPNV treated group only rose from 49.73 on day 1 post infection to 86.67 on day 6 post infection. The difference in IPNV:ELF-1 ratios between the two treatments is surprising as untreated macrophages infected with IPNV would be expected to exhibit equivalent levels of IPNV. Although it is surprising that IPNV:ELF-1 ratios are higher in glucan-treated macrophages than in the untreated controls, virus levels in these experimental groups were not significantly different. This indicated that glucan does not stimulate infected macrophages so as to reduce IPNV levels. This strongly suggests that glucan would not function as an ineffective immunostimulant against IPNV in Atlantic salmon.

5.4.5.2 MAF

The inability of MAF to induce elements of the antiviral response in the macrophages (Figure 31 and Figure 33) was unexpected, since this substance has been shown to be a potent inducer of IFN. There was no significant difference between the Mx:ELF-1 ratios observed in MAF stimulated macrophages and negative controls. Furthermore, there was no significant difference between the IFN:ELF-1 ratios in any experimental group at days 1, 3, 6 and 9 days post infection.

This may be due to the characteristics of the IFN response induced by MAF. The MAF produced by T-lymphocytes stimulated with the T-cell mitogen concanavalin A appears to be similar to IFN-γ (Secombes and Graham, 1990). Thus, the MAF preparations used in this study may have acted so as to induce IFN γ expression in treated

macrophages. IFN γ belongs to the family of type II IFNs, whereas IFNs α and ß are type I IFNs (Levy and Sastre, 2001; Goodburn *et al.,* 2000; Robertsen, 2005). Type I IFNs are known as viral IFNs, whilst type II IFNs are known as immune IFNs. The viral IFNs are, as their name suggests, induced by viral infection, whereas type II IFN is induced by mitogenic or antigenic stimuli (Samuel, 2001). Both types of IFNs induce an antiviral state in target cells through which virus replication is inhibited (Hengel *et al.,* 2005), however as in higher vertebrates, fish Mx transcripts and proteins are typically inducible by type I IFN, poly I:C and virus infection (Robertsen, 2005). In the previous experiment Poly I:C was used to stimulate the macrophages to make the IFN supernatants, which were subsequently able to induce Mx in the isolated Atlantic salmon head kidney macrophages, demonstrating that the IFN secreted by these cells was predominantly type I. The inability of the MAF supernatants in this experiment highlights the specificity of the IFN and Mx responses within Atlantic salmon macrophages in response to stimulation with type I IFN. It is necessary to acknowledge the results of the respiratory burst assays performed in this project, which do not permit verification of the efficacy of the MAF-containing supernatants. However, if it is assumed that the MAF preparations induced a type II IFN response within treated macrophages, we can conclude that such an IFN response is incapable of protecting macrophages from infection with IPNV. This inability to protect macrophages from IPNV infection was reflected in the high IPNV:ELF-1 ratios (Figure 37). The IPNV:ELF-1 ratio rose from 154.63 on day 1 to 473.01 on day 6. However in the control macrophages the IPNV:ELF1 ratio in the only rose from 49.73 on day 1 post infection to 89.91 on day 6 post infection. The difference between these two treatments was significantly different ($P = 0.0001$ day 1 and $P =$

0.0015 day 6). Although it is uncertain why these IPNV levels occurred in infected macrophages, the inability of MAF to prevent infection or induce an Mx response would indicate that this substance is unlikely to prevent IPNV from establishing a carrier state. This raises questions over the potential of MAF as a therapeutic approach to mitigating IPN, as it does not exhibit the ability to prevent infection or reduce the IPNV levels within infected macrophages.

5.4.5.3 LPS

No expression of IFN or Mx was detected in macrophages treated with LPS, or treated with LPS and inoculated with IPNV (Figure 31 and Figure 33). Previous studies of the effects of LPS on fish innate immune responses have yielded conflicting results. Robertsen *et al.,* (1997) detected the expression of Mx by northern blotting in the head kidney and liver of Atlantic salmon intra-peritoneally injected with poly I:C. However, no Mx expression was detected after injection of LPS. Collet and Secombes (2002) could not detect Mx transcripts by RT-PCR in LPS treated RTG cells, in contrast to the efficient induction of Mx by poly I:C in these cells. Johansen *et al.,* (2004) developed an assay for the detection of IFN-like activity in Atlantic salmon based on the transient transfection of CHSE-214 cells with a rainbow trout Mx1 promoter linked to a luciferase reporter. Johansen *et al.,* (2004) tested this by incubating the transfected CHSE-214 cells with supernatants from LPS and Poly I:C stimulated head kidney leucocytes. IFN preparations derived from leucocytes stimulated with poly I:C induced high luciferase expression (greater than 60-fold induction compared to supernatants from non-stimulated cells) in these CHSE-214 cells. However there was no response to supernatants from LPS-stimulated leucocytes, demonstrating the specificity for type I IFN-like activity.

Similarly Robertsen *et al.,* (1997) concluded that Mx genes are induced selectively by double stranded RNA, as LPS or the mere stress of injecting a saline solution did not induce Mx transcription.

However, there have been reports in the literature which indicate that LPS has the ability to induce a type I IFN response in fish. Salinas *et al.,* (2004) detected very low and transient Mx response in the liver of Atlantic salmon parr 2-3 days after administration of *Esherichia coli* (*E. coli*) LPS. However, they suggested that Mx expression may occur in non-hepatic sites, and that liver tissue, which is not rich in macrophages, may not have represented the most appropriate tissue for assessment of the Mx response. Interestingly, Salinas *et al.,* (2004) reported a strong induction of Mx expression in the livers of Atlantic salmon parr following an injection of a commercially available vibrio bacterin, but the bacterial components responsible for this were not investigated. In response to this result Acosta *et al.,* (2004) conducted an experiment to identify the specific components of the bacterin that provides the stimulation. They suggested that the Mx induction may occur in response to LPS or bacterial DNA and subsequently examined the Mx responses of Atlantic salmon to purified *Listonella anguillarum* (*L. anguillarum*) LPS and DNA. They showed that the kinetics of the Mx response to *L. anguillarum* DNA was strikingly similar to that occurring in response to vibrio bacterin and to poly I:C. They suggested that the induction of Mx by *L. anguillarum* DNA is due to the presence of CpG motifs which have been shown to induce Mx expression in Atlantic salmon by Jorgensen *et al.,* (2003). Interestingly Acosta *et al.,* (2004) found that purified *L. anguillarum* LPS induced an Mx response whereas LPS from E. coli and *Salmonella typhimurium* (*S. typhimurium*) failed to so. They suggested that LPS from *L. anguillarum* has unique

effects on the innate immune system not present in LPS from the other species investigated. As the *V. anguillarum* LPS in the experiment described in this thesis failed to induce an Mx response within macrophages, this would agree with the suggestion by Acosta *et al.,* (2004) that *L. anguillarum* has unique effects on the innate immune system not present in LPS from the other species. There are several examples in the literature of studies which fail to demonstrate induction of the type I IFN response by LPS. Johansen *et al.,* (2004) used LPS derived from *E. coli*, whereas, Collet and Secombes (2002) did not specify the origin of the LPS that they used. Whilst Salinas *et al.,* (2004) demonstrated an Mx response within Atlantic salmon using *V. anguillarum* derived LPS, it was not as strong as that observed in experiments using LPS sourced from *L. anguillarum*.

In the present study there was no significant difference between the Mx:ELF-1 ratios occurring in LPS-treated macrophages (Figure 33), LPS treated IPNV-infected macrophages and negative controls. Similarly at 1, 3, 6 and 9 days post infection there was no significant difference between the IFN:ELF-1 ratios occurring in treated macrophages and untreated controls. The inability of LPS to induce an Mx response in macrophages was reflected in LPS-treated, IPNV-infected macrophages. IPNV:ELF-1 ratios fluctuated throughout the period of the study, on day 1 and day 6 the IPNV:ELF-1 ratios (62.15 and 89.91 respectively) were similar to those observed in the control group (day 1, 49.73; day 6, 63.13). However, on days 3 (204.98) and 9 (186.43) the IPNV:ELF-1 ratios rose above those observed in the controls (day 3, 129.90; day 9, 86.67). As the IPNV:ELF-1 ratios in macrophages exposed to LPS were lower than in macrophages exposed to glucan or MAF prior to infection with IPNV, it is possible that LPS is more

efficient at stimulating viral clearance than MAF or glucan. Although it is not possible to completely rule out immunostimulatory effects attributable to LPS based on the results of this thesis, the inability of this substance to reduce the IPNV levels in macrophages between day 1 and day 9 post infection suggest that that LPS is not an effective immunostimulant against IPN. However there is still much to learn concerning the use of LPS as an immunostimulant to mitigate IPN due to the uncertainty surrounding which types of LPS have the ability to induce Mx, and what makes one type of LPS more effective than another.

Whilst the second part of this chapter is concerned with the ability of immunostimulants to mitigate IPNV-infection of Atlantic salmon macrophages, some of the results are relevant to an understanding of macrophage innate immune responses to IPNV. There was no significant difference in the Mx:ELF-1 ratios occurring in IPNV-infected macrophages and those exhibited by negative controls (Figure 33). This finding is in agreement with the lack of an antiviral response in positive control macrophages (i.e. IPNV-infected) in the experiment described in section 5.4.3. The results suggest that IPNV can suppress the type I IFN response in Atlantic salmon head kidney macrophages. Clearly, viruses are unlikely to be successful pathogens if they had not evolved efficient strategies that allow them to suppress IFN production, to down regulate IFN signalling and to block the action of antiviral effector proteins (Haller *et al.,* 2006). There are many reports concerned with the induction of the antiviral state resulting from infection with diverse viruses including IPNV. The results of the present study suggest that IPNV alone does not induce Mx within Atlantic salmon head kidney macrophages. According to

Goodburn *et al.,* (2000) in order to replicate efficiently, it seems likely that all viruses must, at least to a degree, have some means of circumventing the IFN response either by limiting IFN production or blocking IFN actions. The results of the experiments described in sections 5.3.6 and 5.3.7 do not permit definite conclusions to be made to explain the lack of Mx expression in IPNV-infected macrophages. No IFN expression was detected in those macrophages that expressed Mx. Therefore, it would be logical to investigate innate immune responses occurring at the initial stages of infection to determine the timing and manner of the immunosuppression caused by IPNV.

5.5 Conclusion

5.5.1 IFN as a treatment for IPNV

IFN treatment of macrophages prior to infection with IPNV does not prevent infection with this virus. However, the potential may exist for IFN therapy in aquaculture to treat viral diseases such as IPNV because IFN-α/ß is used to treat chronic active hepatitis C or hepatitis B in humans (Samuel, 2001; Sen, 2001). IFN may have therapeutic use as against IPN since macrophages exposed to IFN exhibit enhanced virus clearance. Assuming head kidney macrophages are an important site of virus replication in IPN, IFN could help prevent the establishment of the carrier state. However, it is uncertain what causes IPNV levels to decline in untreated IPNV-infected macrophages, as occurred in Figure 35. This may be due to the existence of antiviral mechanisms in macrophages that are not controlled by the type I IFN/Mx pathway. Alternatively, IPNV may quite simply be destroyed by macrophages.

5.5.2 Immunostimulants as potential treatments for IPN

Development of vaccines against infectious diseases is time consuming and ultimately expensive (Randonic *et al.,* 2004). As a result, it may be more practical to combine good husbandry and the enhancement of disease resistance through the use of immunostimulants to mitigate the effects of infectious diseases such as IPN. There are currently no therapeutic feeds available that are specifically designed to combat viral diseases in fish. Whilst immunostimulants have been proven applicable to aquaculture, the results of the present study suggest that the immunostimulants tested may be better suited to the control of bacterial diseases, as none of those studied were able to induce the antiviral Mx protein. Thus, further studies are required to identify effective immunostimulants for the control of IPN.

5.5.3 The effect of IPNV on the antiviral response within Atlantic salmon head kidney macrophages

In the experiments described in this chapter, expression of Mx or IFN in IPNV-infected macrophages was not significantly greater than that exhibited by uninfected controls. As suggested by McBeath *et al.,* (2007), an improved understanding of the complex host-pathogen relationship in IPN at the molecular level might allow the development of husbandry conditions that favour the host and thus lead to improvements in disease control and fish welfare. The host response to viral infection represents a complex coordination of gene products, which are precisely tuned to activate or inactivate specific pathways and finally counteract the effects of viral gene products (O'Farrell *et al.,* 2002).

According to Rønneseth *et al.,* (2006) viruses interact with immune cells in diverse ways and many viruses act so as to impair or inhibit anti viral responses. It is the opinion of McBeath *et al.,* (2007) that the interaction of IPNV with the IFN system is complex and probably plays a critical role in determining states of resistance or susceptibility in the fish host. The results of this thesis suggest that that IPNV either completely prevents IFN expression or else it blocks IFN from inducing Mx expression. An investigation of IFN expression in the initial stages of infection may elucidate the interaction between IPNV and the innate immune response. This objective is pursued in the following chapter.

Chapter 6 - Comparison of the effects of IPNV and Poly I:C treatment to demonstrate the type I IFN suppressing properties of IPNV in Atlantic salmon head kidney macrophages.

6.1 Introduction

Work described in the previous chapters of this thesis suggests that IPNV does not induce an Mx response in head kidney macrophages of Atlantic salmon. The aim of this chapter is to characterise the antiviral responses within the macrophages at the early stages of infection. The results may lead to a greater understanding of the mechanism through which IPNV inhibits anti-viral responses in macrophages. The expression of IFN in IPNV-infected macrophages will be compared to that induced by Poly I:C. This substance is a potent inducer of type I IFN in mammals and salmonids (Ellis, 2001). From observing the IFN response in IPNV infected macrophages it may be possible to determine whether IPNV has the ability to suppress the IFN response. Despite the fact that the type I IFN system appears to be a potent and efficient mechanism for the host to counteract viruses at early stages of infection, viruses are remarkably successful in infecting their host species. This is probably due to the evolutionary acquisition by viruses of molecular mechanisms which counteract the IFN-α/ß system to allow virus replication (Garcia-Sastre, 2002). Goodburn *et al.*, (2000) summarises some of the major strategies employed by viruses to subvert the IFN system;

i) Inhibition of IFN production

ii) Inhibition of IFN signalling

iii) Inhibition of IFN-induced antiviral enzymes

Experiments conducted in the last chapter (sections 5.3.4 and 5.3.5) demonstrate that neither IFN or Mx are expressed in IPNV-infected macrophages. On the basis of the strategies described by Goodburn *et al.*, (2000), it can be assumed that the strategy employed by IPNV is achieved through inhibition of IFN production or the inhibition of IFN signalling. The sampling points selected in this experiment are designed to detect IFN expression occurring in the early stages of infection. If the results of this experiment show that IFN is expressed in IPNV infected macrophages it can be assumed that IPNV inhibits IFN signalling, thus explaining the inability to detect Mx expression in the previous experiments (section 5.3.4 and 5.3.5). However if no IFN expression is detected in IPNV treated macrophages but Poly I:C induces IFN expression it can be assumed that IPNV inhibits IFN production.

6.2 Materials and Methods

6.2.1 Isolation of macrophages from head kidney

Head kidneys were obtained from Atlantic salmon (section 2.2.2) and macrophages were isolated on 51% percoll as described in section 2.2.4. Macrophages were seeded into 24 well plates at concentrations of 2 x 10^{-7} cells ml^{-1} and maintained at 15°C as described in section 2.2.5.

6.2.2 *In vitro* virus infection in isolated macrophages

After 24 hours incubation at 15°C, macrophage monolayers were processed in triplicate using one of the following three treatments.

a) IPNV-infected group. Macrophages were infected with IPNV at an MOI of 1 (section 2.2.7).

b) Poly-IC-stimulated group. Macrophages were incubated with L-15 medium supplemented with 5% FCS and 50 units ml^{-1} penicillin, 50µg ml^{-1} streptomycin containing Poly I:C (25 µg ml^{-1}).

c) Negative control group. Macrophages were maintained in L-15 medium supplemented with 50 units ml^{-1} penicillin, 50µg ml^{-1} streptomycin and 5% FCS.

6.2.3 RNA extraction and quantitative real-time RT-PCR

After 3, 12, 24 and 36 hours the monolayers were washed three times with L-15 medium and RNA was extracted following the modified TRIzol protocol as described in section 3.2.5. Prior to treatments, RNA was extracted from macrophages to identify the normal expression values for all of the targets investigated in the experiment. This time point was termed “time 0” and the values were incorporated within the Pfaffl relative quantification calculation. The quality and quantity of the extracted RNA was evaluated using a Nanodrop Spectrophotometer. All samples were diluted to 100ng/µl prior to first strand synthesis using RNAse/DNAse free water. Two step RT-PCR was performed on extracted RNA following the steps outlined in sections 4.2.3 and 4.3. All samples were tested for the presence of Mx, IFN and IPNV. The results of the real time amplification of each target was

expressed as a ratio to the internal RT-PCR control ELF-1, using the Pfaffl mathematical equation for relative quantification.

6.2.4 Statistical analysis of real time RT-PCR gene expression

Results are expressed as the relative expression ratio between the target of interest and ELF-1 house keeping gene. Data was analysed on the MINITAB software package by a two way ANOVA, and a Tukey's test was used to perform multiple comparisons to determine the differences between the treatments and time during the course of the experiment. Differences were considered statistically significant when $P<0.05$.

6.2.5 Detection of extracellular IPNV

Prior to extraction of RNA from the macrophage monolayer, the culture medium was tested for the presence of extracellular IPNV as described in section 2.2.8.

6.3 Results

6.3.1 Expression of antiviral genes

IFN expression increased in macrophages treated with Poly I:C (Figure 39). The two-way ANOVA results (Figure 40) show that treatment, time and a combination of treatment and time ($P = 0.001$) all had a significant effect on IFN expression. The tukey test revealed that

after 3 hours treatment with Poly I:C macrophages had significantly higher IFN:ELF-1 ratios than those treated with IPNV (P = <0.0001) and untreated controls (P = <0.0001). Peak IFN expression within Poly I:C treated macrophages occurred at 24 hours post treatment, which was significantly higher than observed 3 hours post treatment (P = 0.0001). IFN expression declined between 24 hours and 36 hours (P = <0.0001). At 36 hours post treatment there was no significant difference in IFN expression between Poly I:C stimulated macrophages and those infected with IPNV (P = 0.9587) or untreated macrophages (P = 0.9995). This result would agree with the opinion of Honda *et al.,* (2005) that the hallmark of mammalian IFN-α/ß is their rapid induction by virus infection as a result of the recognition of viral dsRNA products. In the experiments conducted in the last chapter it would appear that the sampling points may not have been optimal to detect this rapid induction of IFN. Whilst IFN expression increased in macrophages treated with Poly I:C, which is a synthetic double- stranded RNA that is used experimentally to model viral infections, there was no significant difference between the IFN:ELF-1 ratios in IPNV-infected macrophages and negative controls at any of the sample time points, 3 hours after treatment (P = 1.0000); 12 hours (P = 1.0000); 24 hours (P = 0.1578) and 36 hours (P = 1.0000). This suggests that the reason for the inability to detect Mx and IFN expression in IPNV-infected macrophages is a result of the virus blocking IFN production.

IFN induction in the Poly I:C treated macrophages corresponded to an increase in the expression of Mx (Figure 41). The two-way ANOVA results (Figure 42) show that treatment, time and a combination of treatment and time (P = 0.001) all had a significant effect on IFN expression. The tukey test revealed that at 3 hours post-infection, Mx levels in macrophages treated with Poly I:C, or infected with IPNV (P = 0.9241) did not differ from

those exhibited by negative control macrophages (P = 1.0000). However between 3 hours and 12 hours there was a significant increase in Mx expression (P = <0.0001) in Poly I:C treated macrophages, which was maintained up to 24 hours post-treatment (P = <0.0001) when peak levels of Mx expression occurred. IPNV blocked IFN induction in infected macrophages, which did not express Mx at levels greater than those occurring in Poly I:C treated macrophages. Mx:ELF-1 ratios were significantly different in IPNV-infected macrophages and poly I:C treated macrophages at 12, 24 and 36 hours post treatment (P = <0.0001). There was no significant difference in Mx expression in IPNV-infected and negative control macrophages at 3, 12, 24 and 36 (P = 0.9957) hours post infection (P = 0.9101; 1.0000; 1.0000; 0.9957 respectively).

These results strongly suggest that IPNV inhibits the expression of IFN in macrophages and subsequently the induction of Mx. This could explain why IPNV is able to persist within Atlantic salmon head kidney macrophages and establish a carrier state within Atlantic salmon. Furthermore, the kinetics of IFN induction observed in the present experiment confirms that the sampling points studied in chapter 4 were not optimal to detect IFN expression.

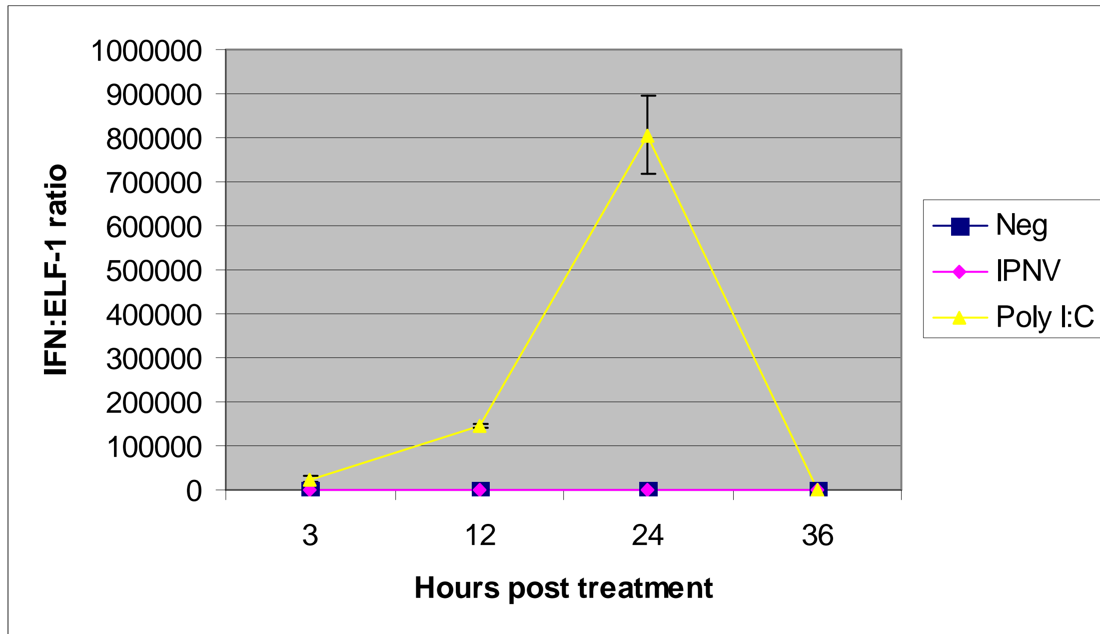


Figure 39: IFN expression in Atlantic salmon head kidney macrophages in response to infection with IPNV or treatment with Poly I:C. Mock-treated macrophages (L-15 alone) served as a negative controls. IFN levels were quantified in relation to expression of the housekeeping gene ELF-1 using the method described by Pfaffl. Data points represent mean IFN:ELF-1 ratios (N = 3) ± SD.

Analysis of Variance for IFN:ELF-1 ratio, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	3	248.627	248.627	82.876	205.21	0.001
Treatment	2	163.146	163.146	81.573	201.98	0.001
Time*Treatment	6	98.345	98.345	16.391	40.59	0.001
Error	24	9.693	9.693	0.404		
Total	35	519.811				

Figure 40: Two way ANOVA results for relative IFN:ELF-1 expressed in (a) IPNV infected; (b) Poly I:C stimulated; (c) untreated Atlantic salmon head kidney macrophages over time. Differences were considered statistically significant when $P<0.05$.

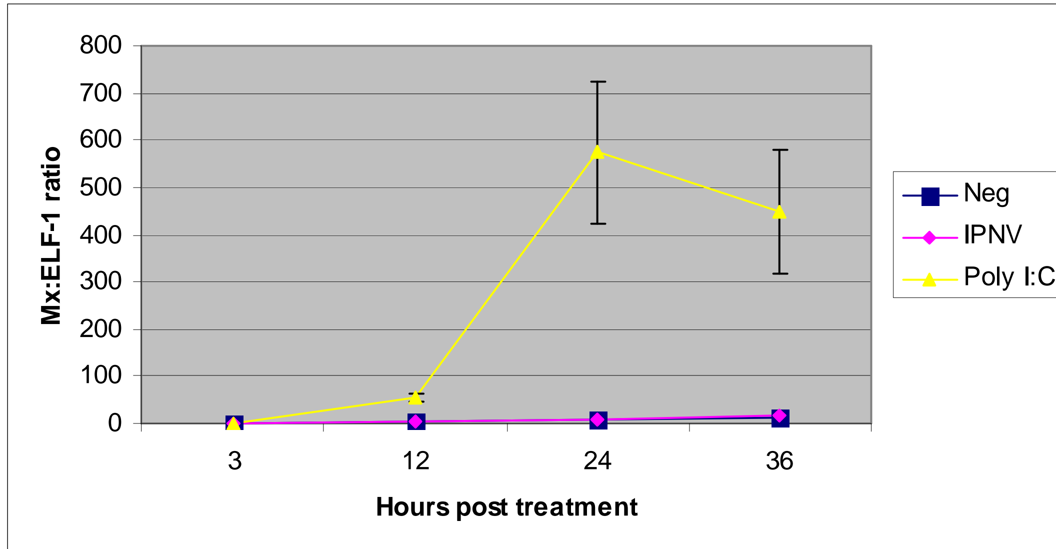


Figure 41: Mx expression in Atlantic salmon head kidney macrophages in response to infection with IPNV or treatment with Poly I:C. Mock-treated macrophages (L-15 alone served as negative controls). Mx levels were quantified in relation to expression of the housekeeping gene ELF-1 using the method described by Pfaffl. Data points represent mean Mx:ELF-1 ratios (N = 3) ± SD.

```
Analysis of Variance for Mx:ELF-1 ratio, using Adjusted SS for Tests

Source          DF    Seq SS    Adj SS    Adj MS       F      P
Time             3    65.277    65.277    21.759  208.57  0.001
Treatmen         2    57.907    57.907    28.954  277.53  0.001
Time*Treatmen    6    24.316    24.316     4.053   38.85  0.001
Error           24     2.504     2.504     0.104
Total           35   150.004
```

Figure 42: Two way ANOVA results for relative Mx:ELF-1 expressed in (a) IPNV infected; (b) Poly I:C stimulated; (c) untreated Atlantic salmon head kidney macrophages over time. Differences were considered statistically significant when P<0.05.

6.3.2 IPNV-levels in infected macrophages

IPNV was detected within infected-macrophages at 3 hours post-inoculation (Figure 43). The two way ANOVA (Figure 44) show that treatment (P = 0.001), time (P = 0.001) and a combination of treatment and time (P = 0.016) had a significant effect on the expression of IPNV. The tukey test revealed that there was an increase in IPNV:ELF-1 ratio between 0 and three hours post-infection (P = 0.0001). The mean IPNV:ELF-1 ratios indicate that there was an increase in the IPNV levels over time, strongly suggesting that IPNV replication occurs in macrophages. Whilst the IPNV:ELF-1 ratio increased from 19007.97 at 3 hours post treatment to 45417.04 at 36 hours post infection, statistical analysis showed that there was no significant difference between the IPNV:ELF-1 ratio observed at 3 hours post infection and 12 hours (P = 0.6575), 24 hours (P = 0.6025) and 36 hours (P = 0.1193). However, IPNV levels at all time points studied were greater than those occurring in macrophages sampled immediately after infection (i.e. time 0, P = <0.0001). Thus, replication of IPNV to a level greater than that contained in the original inoculum did occur, although it is possible that the extent and kinetics of infection differ from those occurring *in vivo*. IPNV was not detected in macrophages treated with Poly I:C or negative controls.

6.3.3 Determination of IPNV levels in the culture media of infected macrophages

No CPE was observed in CHSE-214 monolayers examined over a period 21 days. This indicates that none of the macrophage experimental groups studied released detectable amounts of IPNV into the culture medium. The results of the present study demonstrate that IPNV infection results in the suppression of both IFN and Mx expression. This suppression of the type I IFN response suggests that the ability of macrophages to clear IPNV is

impaired, which may facilitate the establishment of a carrier state that occurs in IPNV-infected Atlantic salmon.

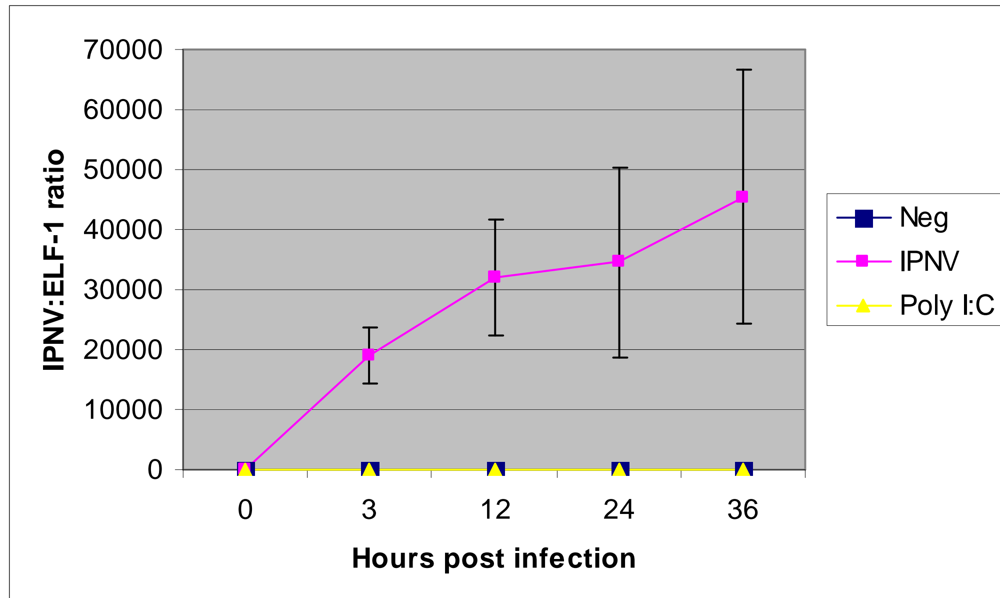


Figure 43: IPNV levels in infected Atlantic salmon head kidney macrophages. Untreated macrophages were used as a negative control. IPNV levels were quantified relative to expression of the housekeeping gene ELF-1 using the method described by Pfaffl. Data points represent mean IPNV:ELF-1 ratios (N = 3) ± SD.

Analysis of Variance for IPNV:ELF ratio, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	3	4.451	4.451	1.484	15.73	0.001
Treatmen	2	679.794	679.794	339.897	3603.94	0.001
Time*Treatmen	6	1.095	1.095	0.182	1.93	0.116
Error	24	2.264	2.264	0.094		
Total	35	687.604				

Figure 44: Two way ANOVA results for relative IPNV:ELF-1 expressed in (a) IPNV infected; (b) Poly I:C stimulated; (c) untreated Atlantic salmon head kidney macrophages over time. Differences were considered statistically significant when $P<0.05$.

6.4 Discussion

6.4.1 Type I IFN response of Atlantic salmon head kidney macrophages to IPNV infection

For a virus to persist, it must actively curtail the host's antiviral immune response (Oldstone, 2006). Little is known about the mechanisms involved in the establishment of carrier states in fish and how viruses evade the host immune defences (Ellis, 2001; McBeath *et al.,* 2007). Understanding the principles by which persistence is initiated and maintained, as well as the pathologic consequences of continued virus replication in a host over its life in terms of causing disease, represents a research area of high significance, and provides opportunities for challenging investigation (Oldstone, 2006). Many viruses have evolved specific mechanisms that antagonize the production or action of IFNs (Goodburn, *et al.,* 2000). As observed in the previous experiments, the inability of IPNV-infected macrophages to express Mx suggested that the virus suppressed antiviral responses within Atlantic salmon head kidney macrophages. In the experiments conducted in chapter 4 (section 5.2.8), due to the experimental design whereby the early stages of infection were not studied, it was not possible to investigate the immediate response of macrophages to the virus. Thus, it was not possible to ascertain where suppression occurred. It is possible that IPNV prevented the induction of IFN; alternatively, it is possible that IPNV infection induces IFN expression, and that the downstream expression of Mx within the macrophage is subsequently blocked. The latter scenario would be in accord with the observations of Collet *et al.,* (2007), who identified high levels of IFN in the supernatant of IPNV-infected macrophage cultures. On the basis of these findings, the sampling points used in the present

study were repositioned to permit analysis of the initial stages of the virus-host interaction, as opposed to sampling at 24 hour intervals over a period of 9 days.

At this point, it is useful to consider the series of events that lead to an antiviral state within a cell following viral infection so as to identify possible points at which suppression may occur during IPNV infection. Figure 45 is a schematic illustration of type I IFN induction in mammalian cells following viral infection. The mammalian IFN-system has been characterised in detail at the molecular level, and the albeit limited number of functional studies that have been performed with the cloned fish IFNs show that they too have characteristic properties of type I IFNs. Virus infected cells synthesize and secrete type I IFNs (IFN-α/ß), which circulate and protect other cells from viral infection (Bergan *et al.*, 2006). During viral infection of mammalian cells, transcription of IFN-ß is induced first through the co-ordinated activation of the transcription factors IFN regulatory factor (IRF) 3 (Robertson, 2005) and nuclear factor kappa B (NF-κ B); (Haller *et al.,* 2006). This “first wave” IFN triggers expression of a related factor IRF-7 (Haller *et al.*, 2006). The induction of IFN-α/ß genes by viruses involves IRF-3 and IRF-7, and expression of the latter is dependant on IFN-stimulated gene factor 3 (ISGF3); (Taniguchi and Takaoka, 2002).

Cells respond rapidly following stimulation with IFN through the JAK-STAT pathway signal inducing pathway (Muñoz-Jordan *et al.,* 2003; Haller *et al.,* 2006). Briefly, the specific receptor complex for each IFN-α/ß and IFN-γ is composed of two major subunits (IFNAR1/IFNAR2 for IFN-α/ß and IFNGR1/IFNGR2 for IFN-γ) and various JAK tyrosine kinases constitutively associated with the receptor. Jak1 and Tyk2 are required for IFN- α/ß signalling; Jak1 and Jak2 are required for IFN-γ signalling (Best *et al.*, 2005). The

signal transducer and activator of transcription (STAT) family of proteins are latent cytoplasmic transcription factors that become tyrosine phosphorylated by the JAK enzymes in response to cytokine stimulation (Samuel, 2001). In the case of signalling via IFN-α/ß, phosphorylated STAT1 and STAT2 bind each other aswell as IFN regulatory factor 9 (or p48), to form the transcription factor (ISGF3g/p48) (Best *et al.*, 2005). This complex translocates to the nucleus and binds to the IFN-stimulated response element (ISRE) in the promoter region of IFN stimulated genes (ISGs) of which some code for antiviral proteins such as Mx (Haller *et al.,* 2006).

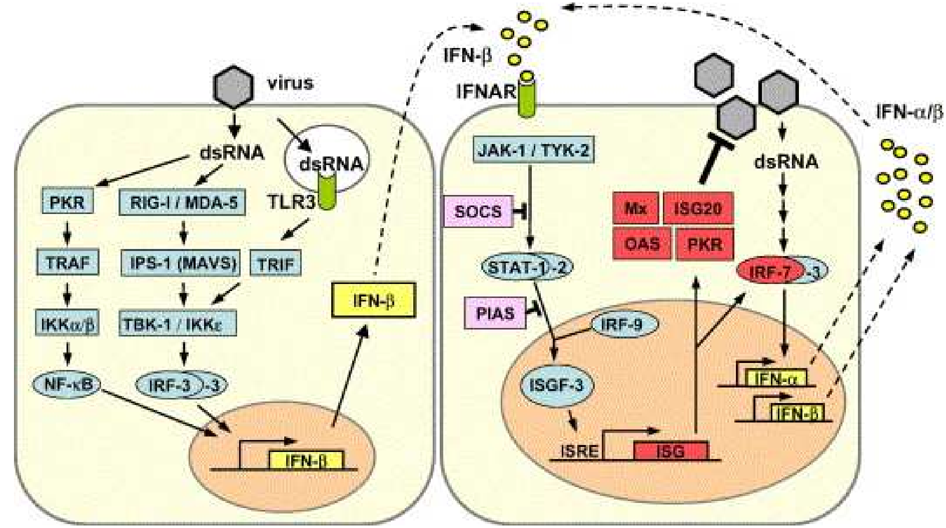


Figure 45: Type I IFN induction, signalling and action. Left panel: dsRNA, a characteristic by-product of virus replication, leads to activation of the transcription factors NF-κ B, IRF-3. The cooperative action of these factors is required for full activation of the IFN-ß promoter. Right panel: Newly synthesised IFN-ß binds to the type I receptor and activates the expression of numerous ISGs via the JAK/STAT pathway which leads to production of intracellular antiviral proteins such Mx protein (from Haller, 2006).

Key

Abbreviation	Term	Abbreviation	Term	Abbreviation	Term
IFNAR	Interferon–α/ß receptor	JAK-1	Janus kinase 1	STAT-2	Signal transducer and activator of transcription-2
IFN- α	Interferon alpha	MDA-5	Melanoma differentiation associated gene-5	TBK-1	TANK-binding kinase-1
IFN- ß	Interferon beta	NK –κB	Nuclear factor kappa B	TLR 3	Toll-receptor 3
IGSF-3	Immunoglobulin superfamily-3	OAS	Oligoadenylate synthetases	TRAF	Tumour necrosis factor associated factor
IKK α/ß	I kappa B kinase	PIAS	Regulator of JAK STAT pathway	TYK-2	Tyrosine kinase 2
IKKε	IκB kinase	PKR	Protein kineases		
IPS-1	Interferon–ß-promoter stimulator 1	PKR	Protein kinase		
IRF-9	Interferon regulatory factor- 9	RIG-1	RNA sensor protein		
ISG	Interferon stimulated gene	SOCS	Suppressors of cytokine signalling		
ISRE	Interferon stimulated response element	STAT- 1	Signal transducer and activator of transcription-1		

According to Garcia-Sastre (2002) the antiviral system is comprised of three main steps

i) detection of viral infection and IFN secretion

ii) binding of IFN to its receptors and transcriptional induction of IFN-stimulated genes

iii) synthesis of antiviral enzymes and proteins which in most cases inhibit key cellular functions upon activation to prevent virus replication

It is helpful to consider the series of events that lead to an antiviral state within a cell and the subsequent strategies that viruses have employed to subvert the IFN response. By doing so the results of this experiment can be used to identify where IPNV subverts the IFN system, thus facilitating its persistence. It has been demonstrated that there is a clear relationship between Mx and the protection of cells against IPNV (Jensen *et al.,* 2002; Larsen *et al.,* 2004). These authors reported that when CHSE-214 cells are either transfected with Poly I:C, an inducer of Mx (Jensen, 2002), or a plasmid containing the Atlantic salmon Mx 1 gene (Larsen *et al.,* 2004), they are protected against IPNV infection.

Poly I:C is a very potent inducer of type I IFN in mammals and type I IFN-like activity in salmonids (Ellis, 2001). For this reason, Poly I:C represents a good positive control for studies of IFN induction, and its potential inhibition by IPNV. Macrophages treated with Poly I:C, exhibited a rapid IFN response (Figure 27) that commenced at between 3 (IFN:ELF1 ratio = 24219.36) and 12 hours (IFN:ELF1 ratio = 144086.2) post treatment, and peaked at 24 hours (IFN:ELF1 ratio = 805768.7). IFN expression decreased to almost undetectable levels at 36 hours post treatment (IFN:ELF1 ratio = 3.75). Since IFNs are unstable proteins that are rapidly degraded (Bergan and Robertson, 2004) it is

probable that the IFN response to a double stranded RNA virus is of short duration. Poly I:C is a synthetic double stranded RNA, as is the genome of the birnavirus IPNV, consequently macrophage responses to IPNV-infection would be expected to share features in common with those occurring in response to poly I:C treatment.

However, in the present study, no IFN expression was detected in IPNV-infected macrophages over the course of the 36 hour sampling period. This strongly suggests that IPNV suppresses macrophage IFN expression *in vitro*. Thus, suppression of macrophage defences by IPNV appears to be achieved through the inhibition of IFN production, as opposed to the inhibition of the IFN-induced antiviral enzymes such as Mx. The difference in IFN levels in IPNV-infected macrophages, and macrophages treated with poly I:C highlights the efficiency of this virus in blocking IFN expression (days 3, 12, 24 $P = <0.0001$).

Goodburn, *et al.,* (2000) have noted that the speed and efficiency of viral suppression of the IFN response may be critical determinants of host range and pathogenicity. As these results would indicate that IPNV has developed a way to block IFN production, it would therefore be interesting to identity the stage at which IPNV blocks IFN production and which part of the virus acts as the IFN antagonist. To be able to replicate efficiently in their hosts, most viruses have acquired genetic information encoding IFN antagonist molecules which block one or more steps of the IFN system (Garcia-Sastre, 2002). Table 7 summarises examples on how specific viruses antagonize the IFN α/ß system.

Table 7: Examples of gene products from different human viruses which have been described to be involved in the inhibition of the IFN-mediated antiviral responses of the host (from Garcia-Sastre 2002).

Virus	Viral IFN antagonist	Pathway targeted
DNA viruses		
Adenovirus	E1A protein	IFN synthesis (IRF-3)
		IFN signaling (IRF-9, STAT1)
	VA RNAs	PKR
Vaccinia virus	B18R protein	IFN signaling (IFNAR)
	E3L protein	PKR
		OAS
		IFN synthesis (IRF-3/7)
	K3L protein	PKR
Herpes simplex virus	ICP34. 5 protein	PKR
	US11 protein	PKR
	Unknown	OAS
Epstein–Barr virus	EBNA-2 protein	IFN signaling
	EBER RNAs	PKR
Cytomegalovirus	Unknown	IFN signaling (JAK1, IRF-9)
Herpesvirus 8	vIRF proteins	IFN synthesis (IRF-1/3/7)
		IFN signaling
		PKR
Human papilloma virus	E6 protein	IFN signaling (TYK2)
	E7 protein	IFN synthesis
Hepatitis B virus	Core antigen	IFN signaling (IRF-9)
	Terminal protein	IFN signaling
Retroviruses		
HIV-1	Tat protein	PKR
	TAR RNA	PKR
	Unknown	OAS
Positive-strand RNA viruses		
Hepatitis C virus	NS5A protein	PKR
	E2 protein	PKR
	Unknown	IFN signaling
Poliovirus	Unknown	PKR
Double-strand RNA viruses		
Reovirus	σ3 protein	PKR
Negative-strand RNA viruses		
Influenza A virus	NS1 protein	IFN synthesis(IRF-3/7, NF-jB)
		PKR
		OAS
Measles virus	Unknown	IFN synthesis
Parainfluenza virus	V protein	IFN signaling (STAT2)
Ebola virus	VP35 protein	IFN signaling (STAT1)
Mumps virus	V protein	IFN synthesis
Parainfluenza virus	Unknown	IFN signaling (STAT1)

Several viruses encode proteins that inhibit IFN synthesis (Sen, 2001). According to Haller *et al.,* (2006) in many cases viruses use non-structural viral proteins to down-regulate IFN responses. This IFN suppression strategy can be exploited in the laboratory to generate

mutant viruses that lack the relevant non-essential proteins. Such viruses still grow in IFN-non-responsive cells but are highly attenuated in IFN-competent hosts. Ferko *et al.,* (2004) demonstrated that modifying the RNA-binding domain of the NS1 protein in influenza A virus, to produce a non replicating NS1 mutant virus, elicited higher levels of IFN-α/ß in serum of immunized mice than the wild-type virus. Similarly, Valarcher *et al.,* (2003) investigated the effects of deletion of the NS genes on the induction of IFN-α/ß by bovine respiratory syncytial virus (BRSV) and their role in establishing BRSV in calves and demonstrated that the NS proteins had a clear role in inhibiting the production of IFN-α/ß. Further results showed that the NS deficient viruses had highly restricted replication within cells; however, immunization induced serum antibodies and protection against challenge with virulent BRSV. Valarcher *et al.,* (2003) concluded that since IFN-α/ß have profound immunomodulatory effects and stimulate the adaptive immune response; it is possible that the greater ability of the NS deficient virus to induce IFN-α/ß may improve the efficacy of vaccines. These results indicate it is possible to produce vaccines lacking proteins with IFN-antagonistic activity (Haller, 2006). This method could therefore provide an interesting area of research for develop of a vaccine for IPNV, which to date has proven difficult; however it is first necessary to identify the IFN-antagonistic structure of IPNV.

Interestingly, all the major essential components of the type I IFN signalling pathway, i.e. IFN-α/ß receptor, JAK1, Tyk2, STAT1, STAT2 and IRF9, have been described as targets for inhibition by viruses (Levy and Garcia-Sastre, 2001). One of the major strategies of viruses for blocking IFN-α/ß production is to target the activities of the IRF transcription factors that bind to the IFN-ß promoter (Goodbourn, 2000). According to Bergan *et al.,* (2006) the role of IRF's in induction of fish IFNs is as yet unknown. In

teleost fish, IRF-1 and IRF-2 have been cloned and sequenced from rainbow trout (Collet and Secombes, 2002) and Japanese flounder (Yabu *et al.*, 1998), whilst an IRF-7 like gene has been cloned from crucian carp (Zhang *et al.,* 2003). Zhang *et al.,* (2003) reported that the carp IRF-7 like gene, similar to human IRF-7, is upregulated by virus infection and IFN treatment and suggested that it plays a critical role in fish IFN signalling and regulation in the expression of IFN-responsive genes. However, studies of IRF-3, which is constitutively expressed in mammalian cells, have not been published for fish (Robertsen, 2005). The importance of the positive feedback loop between the expression of IRF-3 and IRF-7 for the efficient induction of the IFN-α/ß response has been reviewed (Garcia-Sastre, 2002; Taniguchi and Takaoka, 2002; Robertsen, 2005).

Sato *et al.,* (2000) studied the roles of IRF-3 and IRF-7 in mice and showed that both IRF-3 and IRF-7 perform non-redundant and distinct roles from each other. Their results showed that mice cells lacking IRF-3 are more vulnerable to virus infection, whilst cells defective in IRF-7 expression totally fail to induce IFN-α/ß genes in response to infections by any of the virus types they tested. Finally, they demonstrated that a normal induction of IFN-α/ß mRNA could be achieved by co-expressing both IRF-3 and IRF-7, thus proving that together IRF-3 and IRF-7 ensure the transcriptional efficiency of IFN-α/ß genes for the antiviral response. As only two IRF's are cloned in fish more work is required to clone and sequence the components which participate in promoting IFN-α/ß expression. This would therefore enable investigation into the steps in the IFN-α/ß signal transduction pathway that IPNV inhibits to suppress the resulting IFN-α/ß expression. Another interesting target to investigate would be the toll-like receptors (TLR). TLR are a group of transmembrane proteins expressed mainly in dendritic cells (DC) or macrophages (Hoshino

et al., 2002). The activation of cytokine production by TLRs plays an important role in recruiting other components of innate host defence against bacterial pathogens (Tosi, 2005). The mammalian TLR family consists of 10 members (Bricknell and Dalmo, 2005; Tosi, 2005; Plouffe *et al.,* 2005).

Viruses have also been shown to induce a strong activation of cytokine responses mediated by the activation of TLRs (Machida *et al.,* 2006). As Figure 45 shows dsRNA binds and activates the dsRNA activated protein kinase (PKR), however Akira and Hemmi (2003) report that cells dervived from PKR KO mice still respond to the viral RNA mimic Poly I:C, suggesting the existence of another receptor, which recognizes dsRNA. It has been demonstrated that mammalian TLR 3 recognises ds RNA, and that activation of the receptor induces the activation of NF-κ B and the production of type I IFNs (Alexopoulou *et al.,* 2001; Matsumoto *et al.,* 2002). It has also been suggested that TLR 4 is important for the activation for the activation of the innate immune response to viral infection (Haynes *et al.,* 2001; Machida *et al.,* 2006). According to Goodburn (2000) Since the activation of NF-κ B by infection is a key trigger to inducing IFN-α/ß transcription and other immune responses, it would perhaps not be surprising to find that many viruses encoded inhibitors of NF-κ B activation or function.

The results of this experiment confirm that that the positioning of sampling points in previous experiments was not optimal for IFN detection. However due to the close relationship between expression of IFN and Mx (Figure 39 and Figure 41 respectively), it is probable that IFN expression occurred in macrophages expressing Mx. The relationship between IFN expression and Mx induction was clearly demonstrated in this experiment by the close correlation between Mx expression and IFN expression in Poly I:C treated

macrophages. In these cells, peak Mx expression (Figure 41) occurred at 24 hours post treatment (Mx:ELF1 ratio= 573.62) corresponding to peak IFN expression (Figure 39). However, macrophages infected with IPNV did not express IFN and thus would not be expected to exhibit an Mx response. There was no significant difference in Mx expression between the IPNV-infected and controls (3 hours $P = 0.90101$, 12 hours $P = 1.0000$, 24 hours $P = 1.0000$, 36 hours $P = 0.9957$).

Whilst the results of this experiment appear to show conclusively that IPNV inhibits the IFN production in Atlantic salmon head macrophages, this conclusion is in conflict with other published studies of immunity to IPNV infection. The findings of De Sena and Rio (1975) suggested that RTG cells infected with IPNV produced IFN, with similar properties to mammalian and avian IFNs. As this example refers to infections in rainbow trout cell lines, this may suggest that different species, like rainbow trout are more resistant to IPNV infection than others. Whilst this may be true, the cell type investigated in this thesis could provide another explanation for the differences of opinion in literature. It could be hypothesized that the role of the macrophage explains why IPNV may have evolved mechanisms to suppress innate immune functions in this cell. Macrophages are of great importance as scavengers of dead and foreign material (Ellis, 1977). Thus, the macrophage is one of the cell types which a pathogen is likely to encounter upon entry to the host. Given the prominent role of macrophages as effector cells in the immune response, it is not surprising that certain pathogens have evolved mechanisms to promote their survival within these cells. Indeed, it is possible that viruses such as IPNV may exploit macrophages as a shield from other components of the cell-mediated and humoral immune responses

(Kaufmann, 1993). This may ultimately assist IPNV in developing a carrier state in fish populations (Johansen and Sommer, 1995).

The findings of this thesis concur with those of Jensen and Robersten (2002). They demonstrated that the TO-cell line, a cell line which originates from Atlantic salmon head kidney (Wegerland and Johansen, 2001) failed to induce an Mx response when infected with IPNV *in vitro*. On the contrary, Collet *et al.,* (2007) reported the detection of IFN in the supernatant of macrophages from Atlantic salmon following infection with IPNV; however, they also demonstrated that IPNV appeared to suppress Mx expression in RTG cells. In agreement with the findings of Collet *et al.,* (2007) an *in vitro* infection of IPNV was reported to have induced Mx in head kidney leucocytes isolated from rainbow trout (Boudinot *et al.*, 1999). Whilst these results do not agree with the results of this study and those of De Sena and Rio (1975) who reported IFN production in RTG cells, the differences in results could be explained by the nature of the virus. One explanation could be due to differences in the IFN inducing abilities of the strains of IPNV used in these studies. The strain (851/99) used by Collet *et al.,* (2007) may have been more virulent than the one used in this study (975/99). Bruslind and Reno (2000) reported that the differences in amino acid residues located in the VP2 viral capsid protein correlate with the virulence of IPNV isolates. It has been shown that different strains of IPNV induce varying levels of mortality (Bruslind and Reno 2000; Shivappa, *et al.,* 2004; Song *et al.,* 2005); therefore, there may be a link between the differences in virulence between strains of IPNV and the ability of the virus to suppress the innate antiviral response of the host. As previously mentioned NS proteins of viruses have been shown to be IFN antagonists in a range of viral diseases (Joost Haasnoot *et al.,* 2003; Campagna *et al.,* 2005). The method by which RNA viruses replicate

is error-prone (Krönke *et al.,* 2004), and therefore raises the possibility of slight variations in NS proteins occurring amongst the different strains of IPNV viruses which could provide one explanation as to why a conflict exists in the literature as to whether IPNV induces IFN responses in fish cells.

It could be argued that *in vitro* experiments do not give a true representation of the developments *in vivo*. When studying Mx mRNA induction by IHNV (Trobridge *et al.,* 1997) reported a strong expression of Mx following infection *in vivo*, whilst an *in vitro* infection failed to induce a response. They suggested that the induction was not as efficient as in fish tissues, where the presence and interaction of several cell types might lead to more potent induction of IFN. Jensen and Robertsen (2000) reported that two halibut Mx transcripts (2.2 kb and 2.6 kb) were strongly induced *in vivo* by both Poly I:C and IPNV in all organs studied. IPNV has been reported to be capable of inducing an *in vivo* expression of Mx following IPNV challenge (Jensen and Robertsen, 2000; Jensen and Robertsen, 2002; Bergan and Robertsen, 2004; Lockhart *et al.*, 2006). It is possible that *in vivo* experiments report the Mx expression following IPNV infection that is derived from a range of different cell types. On the other hand, the reason why some *in vitro* experiments have demonstrated that IPNV infection fails to induce an Mx response may be from the use of cell types in which the virus is able to block the IFN response. If IPNV does exploit macrophages as a shield from components of the cell-mediated and humoral immune responses as Kaufmann (1993) suggests is possible, then this is could explain how fish that exhibit an Mx response become IPNV carriers.

As the results of this thesis suggest that IPNV suppresses IFN production in Atlantic salmon head kidney macrophages, and the results of chapter 4 reveal that stimulation of

macrophages with IFN (section 5.3.6) prior to infection aids the reduction of intracellular viral levels, IFN therapy may represent a logical treatment to investigate as a means to treat IPN. IFN therapy has been used to treat a range of viral diseases in humans; IFN-α/ß is clinically used to treat chronic active hepatitis caused by hepatitis C or hepatitis B viruses (Samuel 2001; Sen, 2001). However Goodburn *et al.,* (2000) suggest that the ability of viruses to block the IFN response may have consequences in relation to chronic or persistent viral disease. Thus, while initially it may appear to be the most logical choice, IFN may not represent an effective means of treatment of some chronic virus infections because viruses have mechanisms for circumventing the IFN response. The findings of Lockhart *et al.,* (2004) agree with this suggestion, as IPNV continued to persist for 14 days, following an injection of Poly I:C, in naturally infected Atlantic salmon. Poly I:C administration was shown to successfully induce Mx, suggesting that induction of an IFN response with poly I:C is probably not a feasible means of treating Atlantic salmon broodstock that are also IPNV carriers. However, although IPNV has been shown to have the ability to circumvent the type I IFN response in Atlantic salmon head kidney macrophages, the results of the previous chapter showed that if the antivirial response was triggered in the cell prior to infection, then the IFN treatment appears to have a significant effect on reducing the level of virus in the infected macrophage.

In fish, there have been other positive results in terms of activating the innate immune response. Jorgensen *et al.,* (2001) demonstrated that non-methylated CpG DNA induces production of antiviral cytokines in adherent salmon head kidney leucocytes, and suggested that the immune system recognises unmethylated CpG motifs as a “danger signal” which subsequently activates the immune system. Following this, Jorgensen *et al.,*

(2003), reported that Atlantic salmon treated with CpG DNA prior to challenge with IPNV exhibited reduced viral titres and lower mortality compared to controls. Consequently, CpG DNA could be used as an adjuvant or immunostimulant with the aim of reducing IPNV-associated mortality.

6.4.2 Intracellular levels of IPNV following infection in Atlantic salmon macrophages

IPNV:ELF-1 ratios of macrophages inoculated with IPNV were examined to ensure that the virus successfully infected the cells. Figure 43 demonstrates unequivocally that there was an increase in IPNV:ELF-1 ratio between 0 and three hours post-infection (P = 0.0001). IPNV levels also increased between three hours (19007.97) and 36 hours (45417.04) post infection. However this increase was not significantly different (P = 0.116). Experiments in the previous chapter (sections 5.3.6 and 5.3.7) demonstrate that IPNV levels did not markedly increase throughout the duration of the experiment. This suggests why IPNV is described as harbouring within Atlantic salmon head kidney macrophages (Johansen and Sommer, 1995). This may enable IPNV virus to persist within its salmonid host and maintain a life long carrier status within the fish.

6.5 Conclusion

The results of this study are in agreement with published studies which demonstrate that teleosts possess an innate antiviral defence system, incorporating the type I IFN system. This includes IFN-induced effector proteins such as Mx. Poly I:C was found to be a good inducer of IFN, whilst IPNV appears to have evolved a strategy to avoid the type I IFN

defence system of Atlantic salmon, possibly facilitating persistence within macrophages. The results suggest that IPNV circumvents the antiviral immune response in Atlantic salmon head kidney macrophages by blocking IFN production. In Poly I:C treated macrophages IFN expression peaked at 24 hours post treatment and then immediately declined to undetectable levels. The inability to detect IFN at later time points confirmed that the sampling points used in initial experiments were unsuitable for detection of the IFN response. Blocking of IFN production would explain why macrophages and peripheral monocytes are a target cell for IPNV in persistently infected fish (Collet *et al.*, 2007). The results of this study appear to be in contrast with other studies that demonstrate IPNV has an ability to induce Mx. However, these studies were performed in different types of cell which may have the ability to induce Mx in response to IPNV. There is also the likelihood that different strains of IPNV have varying IFN antagonistic properties. The results presented in this thesis support McBeath *et al.,* (2007) contention that induction of the IFN system by IPNV involves complex virus/host interactions and may play a role in determining states of resistance or susceptibility. With further work, it would be possible to characterise IPNVs IFN antagonism in more detail, and to confirm the stage of the IFN response that this virus blocks in order to suppress the antiviral response. A better understanding of the IFN antagonistic properties of viruses such as IPNV would be of great benefit for the rational design of novel live, attenuated viral vaccines and holds the promise of providing novel targets for development of antiviral compounds active against human and animal pathogens (Levy and Garcia-Sastre 2001).

Chapter 7 - General discussion

7.1 Discussion

7.1.1 Macrophage culture and real time RT-PCR assay development

The overarching objective of this project was to study the effects of IPNV on the innate immune response of Atlantic salmon macrophages. The close relationship of IPNV to Atlantic salmon head kidney macrophages (Johansen and Sommer, 1995) made the macrophage a logical choice for *in vitro* experiments. Macrophages also play a major role in innate and adaptive immune response (Sørensen *et al*., 1997). IPNV can be detected within the macrophages of persistently infected carrier fish (Sadasiv, 1995). An understanding of host immune responses to IPNV is crucial for the development of effective vaccines to counter this virus. IPNV vaccines must not only protect against disease, but also prevent the development of infectious carriers.

Consequently, characterisation of the immune mechanisms involved in the generation and maintenance of the carrier-state are of great importance for the design of IPNV vaccines and ultimately for the control of IPN. Vaccines that simply reduce or eliminate mortalities will do little to solve the problem of IPN on a worldwide scale (Reno, 1999). In the present study, a reproducible technique was developed for the *in vitro* culture of macrophages for up to 12 days post-isolation (chapter 2). In conjunction with this a reliable RNA extraction method was developed which facilitated quantitation of immune related gene expression by real time RT-PCR for IPNV, IFN and Mx relative to the "housekeeping gene" ELF-1 (Chapter 3). Limitations were imposed on this study by the

yields of macrophages obtained from salmon head kidneys, and the inability to maintain cultures in 96 well plates. This restricted the number of replicates that could be studied in IPNV infection experiments, and subsequently limited the statistical power of experiments. Ideally, greater numbers of replicates should be studied, but financial and logistical constraints prevented such an approach in the present study. However as the effects of many of the experimental manipulations were pronounced, this drawback did not negate the overall conclusions of the project. In future studies, a larger consumables budget and more manpower, would permit the use of experiments with more time points and the investigation into further mRNA targets.

7.1.2 Innate immune responses in IPNV-infected Atlantic salmon head kidney macrophages.

The effect of IFN on IPNV levels in infected macrophages was investigated in chapter 4. The efficacy of the IFN preparations was confirmed in a luciferase reporter assay. Since a relationship has been demonstrated between Mx expression and protection against IPNV (Nygaard *et al.,* 2000) it was anticipated that IFN-treated macrophages would be more efficient in clearing the virus. The findings of this experiment would compliment the other findings of this thesis. It was demonstrated that stimulation of macrophages prior to infection helped reduce the viral levels in infected cells (section 5.3.6). As IPNV has evolved an ability to suppress the type I IFN system in Atlantic salmon macrophages there is a need to identify and develop therapeutic means of activating the macrophage's type I IFN system prior to or in the presence of IPNV infection. The ability of immunostimulants to prevent infection or aid the clearance of IPNV from infected macrophages was therefore

investigated in the present study. Three immunostimulants, MAF, glucan and LPS, were tested for the ability to up-regulate the innate immune response of the Atlantic salmon macrophage (section 5.3.5). Immunostimulants increase resistance to infectious disease, not by enhancing specific immune responses, but by enhancing non-specific defence mechanisms (Sakai, 1999). All three treatments studied have been shown to be effective against bacterial diseases. However, in the present study, none were able to induce Mx expression, and thus they may not represent useful means of controlling IPNV. The inability to induce an Mx response may be due to the specificity of type I IFN signalling. If the immunostimulants were shown to have the ability to enhance virus clearance this could have potentially helped prevent the establishment of carrier status, which would consequently help to reduce the incidence of IPN in wild and farmed fish. The lack of published studies reporting successful use of immunostimulants to mitigate virus infections in aquaculture suggests that they may only have a limited use.

7.1.3 IPNV levels in Atlantic salmon head kidney macrophages

IPNV levels were investigated in macrophages following infection to ascertain if replication of the virus occurred within the cell. Following the development of a reproducible *in vitro* culture technique, it was demonstrated that Atlantic salmon head kidney macrophages can be successfully infected *in vitro* (section 5.4.1). There was a significant increase in the infected macrophage virus levels between the time 0 and 1 day post infection sample point. Infected macrophage virus levels were significantly greater than in uninfected cells at all time points. This result would suggest that IPNV has the ability to replicate in Atlantic

salmon head kidney macrophages during the early stages of infection. Peak virus levels occurred between 3 and 5 days post infection (Figure 24).

Whilst increases in the mean IPNV:ELF-1 ratios were observed in the infected macrophages, none of these increases were shown to be significant between 1 and 9 days post infection. Whilst the experiment demonstrates that IPNV infects macrophages *in vitro*, replication of the virus may occur at relatively low levels. IPNV levels declined between 5 and 9 days post infection, but crucially the virus was not completely cleared, as levels at 9 days post infection were still significantly greater than in uninfected cells. This observation is in agreement with the persistent infections reported elsewhere.

The results of the present study are not in agreement with several studies that suggest that IPNV persists within macrophages without replicating (Nova *et al.,* 1996, Munro *et al.,* 2006, Collet *et al.,* 2007). However, the present study does suggest that the macrophage is not the cell in which the bulk of virus replication occurs in IPN of Atlantic salmon, as originally proposed by Yu *et al* (1982). It should be emphasised that the experiments conducted in this thesis have focused on IPNV infection of macrophages *in vitro* infection, and thus it would be interesting to compare the results with *in vivo* studies of the IPNV infection.

In IFN treated IPNV-infected macrophages, IPNV levels were significantly reduced during the course of the experiment, suggesting that IFN may represent a potential treatment for IPN (Figure 35). However, IPNV levels also declined in IPNV-infected untreated macrophages. Whilst the reduction in IPNV levels was not statistically significant, this result suggests that other components of the immune response may be responsible for the declining virus levels. However, as infected fish normally develop a life long

asymptomatic carrier state it is not likely, if there is any alternative antiviral response, that it is of great significance as it is not capable of clearing the virus entirely. Nevertheless, this again highlights the advantage of a larger number of genes or novel mRNA targets being investigated in order to give a more in depth picture of the relationship between IPNV and the head kidney macrophage. For a clearer insight into how IPNV replicates in head kidney macrophages, qRT-PCR utilising absolute quantification would provide more rigorous results than the relative quantification method used in the present study. Laminarin, *V. anguillarum* LPS and MAF were found to be incapable of inducing Mx, and therefore may be better suited to the control of bacterial diseases.

The absence of IPNV in macrophage culture media assessed by infectivity assays in CHSE-214 cells suggests that any decline in IPNV levels is a result of the virus being broken down in macrophages rather than shed. However, it is possible that washing of the macrophage monolayers and replacing of the culture medium may have led to the removal of any virus shed by the cells.

7.1.4 Suppression of type I IFN response in Atlantic salmon macrophages by IPNV

In order to develop effective vaccines it is helpful to have an understanding of the host-pathogen relationship. Many fish viral pathogens establish a persistent carrier state in the host; however little is known about the mechanisms involved in the establishment and maintenance of viral carrier states in fish and how viruses evade the hosts defences (Ellis, 2001). As the innate immune system is the first line of defence against viral infections, and as the response of this system is primarily through induction of type I IFN's and the activation of NK cells, viruses frequently act so as to subvert one or more of these

mechanisms to prolong survival (Rønneseth *et al.,* 2006). The findings of the present study suggest that IPNV targets the type I IFN system, and this may facilitate the establishment of a carrier state. More specifically the results presented in Chapter 5 (section 6.3.1) demonstrate that IPNV has evolved the ability to block IFN production. No expression of IFN or Mx occurred in IPNV-infected macrophages in the experiments conducted in Chapter 4 (sections 5.3.4 and 5.3.5) and since Mx expression occurred in the Poly I:C treated macrophages (which serve as a positive control), it is probable that this was due to IPNV-mediated immunosuppression. Unfortunately, the sampling points in experiments conducted in chapter 4 were not optimal to detect IFN expression, and consequently no definite conclusions could be made concerning how IPNV suppresses the type 1 IFN response. However, through the use of sampling points positioned earlier in the course of infection, it was possible to demonstrate that IFN production was blocked by IPNV as opposed to IFN signalling (Figure 39). Further characterisation of the mechanism through which IPNV prevents IFN production would be of great benefit to the development of an IPNV vaccine, as it may be possible to generate attenuated viruses by altering specifically those gene(s) responsible for inhibition of IFN function (Goodbourn *et al.,* 2000).

In summary, methods for the *in vitro* cultivation of macrophages and quantitative RT-PCR were used to characterise innate immune responses in IPNV-infected Atlantic salmon macrophages. The results of this research suggest that IPNV may block IFN production rather than IFN signalling. Three immunostimulants (MAF, LPS and glucan) were ineffective in boosting macrophage innate immune responses to IPNV. These results highlight potential means to control IPN through rational design of attenuated IPNV vaccines.

7.1.5 Relevance of findings to the current status of IPN in Scotland

Persistent IPNV infection of Atlantic salmon poses a significant and costly threat to the aquaculture industry. Scottish salmon production in 2000 exceeded 130,000 tonnes and, at £400M, contributed up to 40% of Scottish food exports (Rae, 2002). According to Murray (2006 b) most of this production occurred in areas with few economic alternatives, therefore losses of salmon production due to IPN are of considerable importance. IPN is an example of a disease which is emerging in the aquaculture of finfish. This disease was first reported from trout hatcheries in North America in the 1950s (Wood *et al.*, 1955) but has since spread to most countries with salmonid production. Hence, the results of the present study are particularly timely. Moreover, recent legislative changes have increased the requirement for effective means of controlling IPN. On commencement of this study IPN was categorised as a List III disease under Annex A of European Union (EU) council directive 91/67. Therefore in Great Britain IPN was a notifiable disease under The Diseases of Fish Acts 1937 and 1983. A recent assessment has identified IPNV as an increasingly common pathogen in farmed Scottish Atlantic salmon; with over 80% of marine sites are now infected (Murray, 2006 a). As a result of this increase in prevalence, during the course of this study IPN was deregulated from a List III disease to a non notifiable disease rating. IPN is now so widespread, the threat of IPNV infection to Atlantic salmon is of great significance for the farmer, and subsequently increases the need for them to protect their fish. It is a widely held opinion that the only effective way to control virus infections in aquaculture is to prevent the exposure of fish to pathogenic viruses and especially to prevent movement of infected fish between farms (Blake *et al.*, 1995; Williams *et a*l., 1999; Milne, 2006 a). However, as the virus is so widespread there is little that can be done by the

farmer to prevent his stock coming into contact with the virus. A stark reality of this is the susceptibility to an outbreak of IPN in Atlantic salmon post-smolts shortly after seawater transfer (Rønneseth *et al.,* 2006). This therefore increases the importance of understanding the immunity of Atlantic salmon with respect to IPNV infection, and subsequently undertaking research into how the immune system can be enhanced. Possible steps to limit the consequences of IPN might involve the improvement of disease resistance through breeding programmes, and modulation of the immunity through vaccines, immunostimulants or other means.

To put the findings of this study in context with respect to the current status of IPN in Scotland, there are many areas of work that require attention. It is the opinion of Murray (2006 a) that in practice eradication of IPNV from Scotland now seems impractical. This study appears to be the first in the literature to demonstrate that IPNV specifically blocks the production of IFN within Atlantic salmon macrophages. Further characterisation of the mechanism of IFN inhibition by IPNV would assist with the development of control measures. In terms of making fish more resistant to IPNV the most obvious solution would be vaccination to make the post-smolts less susceptible to infection. According to the report of the Aquaculture Health Joint Working Group on Infectious Pancreatic Necrosis Virus in Scotland published by the Scottish Executive, several vaccines against IPNV are currently undergoing field trials in marine sites in Scotland, Norway and Chile. However, the efficacy of these vaccines in protecting against mortality in post-smolts is still uncertain because of the lack of reliable lethal challenge models. In addition, the development of vaccines against infectious diseases is time consuming and ultimately expensive (Randonic *et al.*, 2004). It may also be difficult to administer vaccines to salmon at the fry life cycle stage. It

is hoped that results as presented in this thesis will increase the understanding of IPN, however there are still many more questions still to be answered concerning the persistence of IPNV within the fish. Moreover, since there are no effective treatments available against IPN more research is required. For example, further work is essential to characterise the Atlantic salmon innate immune response. Effective treatments against IPN are vital to enable economically viable culture of Atlantic salmon to continue in an area where eradication of the virus seems impractical. A number of potential approaches are discussed in the ensuing future work section.

7.2 Future work

In this thesis, a technique was developed for the culture of macrophages and the subsequent extraction of RNA for use in real time RT-PCR analyses of immune targets. This enables an opportunity for reproducible *in vitro* studies of macrophage immunity to IPNV. In the present study Mx and IFN have been investigated together with IPNV levels in infected macrophages. Other molecular biological methods could also be applied to the study of macrophage immunity to IPNV. For example, microarray technology provides a powerful tool for measuring the expression levels of large numbers of genes simultaneously and creates unparalleled opportunities to study complex physiological or pathological processes, including the development of disease, that are mediated by the co-ordinated action of multiple genes (Kerr *et al.,* 2000). Detection of genes differentially expressed across experimental, biological, and/or clinical conditions is a major objective for microarray experiments (Tan *et al.,* 2006). Microarray analyses of IPNV-infected macrophages and

controls would represent a powerful means of characterising the immune response to an important fish pathogenic virus.

The results of the present study were based entirely on *in vitro* experiments. As discussed above, the results of this thesis are in conflict with other studies, with respect to the ability of IPNV infected macrophages to express Mx. Whilst all attempts were made to provide the optimal culture conditions for the macrophages, an *in vitro* experiment can never fully reproduce *in vivo* conditions. Therefore it would be of interest to conduct an experiment where Atlantic salmon are challenged *in vivo* with IPNV, with head macrophages sampled over a time period post-infection. The expression of IFN and Mx within these macrophages could be compared to controls and also macrophages infected *in vitro*. This would reveal whether isolation and *in vitro* culture effect macrophage gene expression.

It would also be interesting to investigate the interaction between IPNV and other cell types. According to Rønneseth *et al.,* (2006) the role of neutrophils and their regulation during infections in fish is poorly understood. These authors demonstrated that neutrophils are involved in virus clearance and are affected by IPNV for weeks after the commencement of infection. This again raises the important question, how well do *in vitro* studies represent what is happening within a fish? By building on the knowledge gained in this study regarding the innate immune response of Atlantic salmon head kidney macrophages in response to IPNV, it would be possible to investigate the IFN suppressing abilities of IPNV in other cell types. By identifying all the possible sites of virus persistence, it would enable a greater understanding of the carrier state.

The most important area of work that requires further research as a consequence of the findings of this study concerns the ability of IPNV to block IFN production in Atlantic salmon head kidney macrophages. With further work, it would be possible to characterise more comprehensively the interaction between IPNV and host encoded proteins. One possible method of identifying which structure of IPNV is responsible for blocking the IFN-α/ß response in Atlantic salmon head kidney macrophages would be RNA interference (RNAi). In recent years, sequence-specific gene silencing has been an area of increasing focus, both because of its interesting biology and because of its power as an experimental tool (Denli and Hannon, 2003). In animals and protozoa gene-specific double-stranded RNA triggers the degradation of homologous cellular RNAs, the phenomenon of RNAi. RNAi has been shown to represent a novel paradigm in eukaryotic biology and a powerful method for studying gene function (Ullu *et al.,* 2002). Target genes can be silenced by transfection of chemically or enzymatically synthesized small interfering RNAs (siRNA) or by DNA based-vector systems that encode short harirpin RNAs (shRNAs) that are further processed into siRNAs in the cytoplasm (Du *et al.,* 2006). Campagna *et al.,* (2005) designed 19bp siRNAs to target the genome segment 11 of two strains of Rotavirus to demonstrate that NSP5 is an essential protein for the formation of viroplasms and for virus replication. Both siRNAs were entirely specific and they abolished the expression of NSP5 through the knockdown of segment 11 RNA. It has been suggested that as RNAi has been shown to play a role in viral clearance studies (Kapadia *et al.,* 2006; Yoon, 2004; Wang, 2004), therapeutic induction of RNAi either alone or in combination with IFN treatment might represent an alternative approach for the treatment of chronic diseases (Kapadia *et al.,* 2006). Inhibition of virus replication by means of induced RNAi have been reported for

numerous viruses, including several important human pathogens such as human immunodeficiency virus type 1, hepatitis C virus, hepatitis B virus, dengue virus, poliovirus and influenza virus A. Examples of these are reviewed in Joost Haasnoot *et al.,* (2003). However, Krönke *et al.,* (2004) have raised the important point that the error-prone replication of RNA viruses, which gives rise to the rapid evolution of escape mutants, may represent an obstacle for the development of siRNA-based gene therapies. For this reason, they suggest that RNA viruses and retroviruses will be especially difficult to eradicate.

Once the viral structure responsible for the blocking of the IFN- α/ß response is identified, it would be possible to examine the direct effect of the interaction between the antagonist and the various steps in the IFN pathway. Through doing this, the specific stage at which the virus blocks the production of IFN would be revealed. A commonly used technique that has been used to facilitate the study of protein-protein interactions is the yeast two-hybrid system (Fields and Song, 1989). The system is based on the ability to split a transcription factor (GAL4) into two separable functional domains: a DNA-binding domain and a transcriptional activation domain. Each one when expressed separately is unable to activate transcription. These domains are used to generate hybrid proteins to be tested for potential protein-protein interaction. Plasmids encoding two hybrid proteins one consisting of the GAL4 DNA-binding domain fused to the "bait" protein and the other consisting of the GAL4 activation domain fused to the "prey" protein are constructed and introduced into the yeast (Chien *et al.,* 1991). Once "bait" protein - "prey" protein interaction is generated, it reconstitutes a functional transcription factor that can be readily monitored using reporter gene assays in yeast (Aronheim, 2000). However according to Shioda *et al.,* (2000) some interactions of mammalian proteins may not occur in the yeast

milieu because of possible lack of associating factors, protein modifications (such as signal-induced phosphorylation), or correct protein folding. As a result, Shoida *et al.,* (2000) developed a mammalian cell two-hybrid screening system to identify interacting proteins that are difficult to detect by the yeast system. However, these methods are restricted in the fact that they do not cater for transcription factor interactions and will only indicate if protein interaction has occurred. As this method only identifies interactions between proteins it would therefore only be possible to investigate which proteins corresponding to the numerous stages of the IFN pathway the IPNV antagonistic structure interacts with. Subsequently one is only able to propose that IFN blocking may occur at this stage as the yeast 2 hybrid method does not demonstrate how this interaction affects the outcome of the pathway.

As two-step real time RT-PCR has been implemented to analyse the expression of targets in this thesis from the extracted macrophage RNA, a library of cDNA which can be kept indefinitely (Bustin, 2000, Peters *et al.*, 2004), is generated for each experiment. This is a considerable advantage as other mRNA targets can be quantified with relative ease when new and interesting targets are detected in fish. With investigation into further targets it would be possible look for alternative targets within the macrophage IFN system which are affected by IPNV infection, and subsequently this may be another way of highlighting the specific stage at which the virus blocks IFN production. Thus, the cDNA stocks produced in this project represent a useful resource for further study of macrophage immunobiology.

References

Acosta, F., Lockhart, K., Gahlawat, S.K., Real, F. and Ellis, A.E. (2004). Mx expression in Atlantic salmon (*Salmo salar* L.) parr in response to *Listonella anguillarum* bacterin, lipopolysaccharide and chromosomal DNA. *Fish and Shellfish Immunology*, **17**, 255-263.

Ahmet, M.D. and Hannon, G.J. (2003). RNAi: an ever-growing puzzle. *Trends in Biochemical Sciences*, **28**, 196-201.

Ahne, W. and Negele, R.D. (1985). Studies on the transmission of infectious pancreatic necrosis virus via eyed eggs and sexual products of salmonid fish. In: Ellis, A.E., (Ed.) *Fish and Shellfish Pathology,* pp. 261-269. London: Academic Press.

Ahne, W., Kelly, R.K. and Scholtfeldt, H.J. (1989). Factors affecting the transmission and outbreak of infectious pancreatic necrosis (IPN). In: Lillelund, K. and Rosenthal, H., (Eds.) *Contributions to the Canadian-German cooperation programme: "Fish health protection studies"*, Hamberg.

Akira, S. and Hemmi, H. (2003). Recognition of pathogen-associated molecular patterns by TLR family. *Immunology Letters*, **85**, 85-95.

Alexopoulou, L., Holt, A.C., Medzhitov, R. and Flavell, R.A. (2001). Recognition of double-stranded RNA and activation of NF-κ B by Toll-like receptor 3. *Nature*, **413**, 732-738.

Alonso, M., Rodriguez, S. and Prieto, S.I.P. (1999). Nested PCR improves detection of infectious hematopoietic necrosis virus in cells co infected with infectious pancreatic necrosis virus. *Journal of Virological Methods,* **81**, 1-9.

Altmann, S.M., Mellon, M.T., Distel, D.L., Kim, C.H. (2003). Molecular and functional analysis of an interferon gene from the zebrafish, *Danio rerio*. *Journal of Virology*, **77**, 1992-2002.

Anderson, D.P. (1992). Immunostimulants, adjuvants and vaccine carriers in fish: Applications to aquaculture. *Annual Review of Fish Diseases*, **2**, 281-307.

Anon. (2003). Final report of the Aquaculture Health Joint Working Group subgroup on infectious pancreatic necrosis in Scotland. Aberdeen: Fisheries Research Services. 90p.

Ariel, E. and Olesen, N.J. (2002). Finfish in aquaculture and their diseases - A retrospective view on the European Community. *Bulletin of the European Association of Fish Pathologists,* **22**, 72-85.

Aronheim, A. (2000). Protein Recruitment Systems for the Analysis of Protein-Protein Interactions, *Biochemical Pharmacology*, **60**, 1009-1013.

Barlic-Maganja, D., Strancar, M., Hostnik, P., Jencic, V. and Grom, J. (2002). Comparison of the efficiency and sensitivity of virus isolation and molecular methods for routine diagnosis of infectious haematopoietic necrosis virus and infectious pancreatic necrosis virus. *Journal of Fish Diseases,* **25**, 73-80.

Bebak, J., Mcallister, P.E. and Smith, G. (1998). Infectious pancreatic necrosis virus: transmission from infectious to susceptible rainbow trout fry. *Journal of Aquatic Animal Health,* **10**, 287-293.

Bergan, V. and Robertsen, B. (2004). Characterization of Atlantic halibut (Hippoglossus hippoglossus) Mx expression. *Developmental and Comparative Immunology,* **28**, 1037-1047.

Bergan, V., Steinsvik, S., Xu, H., Kileng, Ø. and Robertsen, B. (2006). Promoters of type I interferon genes from Atlantic salmon contain two main regulatory regions. *FEBS Journal*, **273**, 3893-3906.

Best, S.M., Morris, K.L., Shannon, J.G., Robertson, S.J., Mitzel, D.N., Park, G.S., Boer, E., Wolfinbarger, J.B. and Bloom, M.E. (2005). Inhibition of Interferon-Stimulated JAK-STAT

Signalling by a Tick-Borne Flavivirus and Identification of NS5 as an Interferon Antagonist, *Journal of Virology*, **79**, 12828-12839.

Biering, E. and Bergh, O. (1996). Experimental infection of Atlantic halibut, *Hippoglossus hippoglossus* L, yolk-sac larvae with infectious pancreatic necrosis virus: Detection of virus by immunohistochemistry and in situ hybridization. *Journal of Fish Diseases,* **19**, 261-269.

Biering, E. (1997) Immune response of the Atlantic halibut (*Hippoglossus hippoglossus* L) to infectious pancreatic necrosis virus (IPNV). *Fish and Shellfish Immunology,* **7**, 137-149.

Biering, E., Villong, S., Sommerset, I., Christie, K.E. (2005) Update on viral vaccines for fish. *Developments in Biologicals*, **121**, 97-113.

Bird, S., Zou, J., Savan, R., Kono, T., Sakai, M., Woo, J. and Secombes, C. (2005). Characterisation and expression analysis of an interleukin 6 homologue in the Japanese pufferfish, *Fugu rubripes*. *Developmental and Comparative Immunology*, **29**, 775-789.

Blake, S.L., Schill, W.B., Mcallister, P.E., Lee, M.K., Singer, J.T. and Nicholson, B.L. (1995). Detection and identification of aquatic birnaviruses by PCR assay. *Journal of Clinical Microbiology,* **33**, 835-839.

Blake, S., Ma, J.Y., Caporale, D.A., Jairath, S. and Nicholson, B.L. (2001). Phylogenetic relationships of aquatic birnaviruses based on deduced amino acid sequences of genome segment A cDNA. *Diseases of Aquatic Organisms,* **45**, 89-102.

Bootland, L.M., Dobos, P. and Stevenson, R.M.W. (1991). The IPNV carrier state and demonstration of vertical transmission in experimentally infected brook trout. *Diseases of Aquatic Organisms,* **10**, 13-21.

Bootland, L.M., Dobos, P. and Stevenson, R.M.W. (1990). Fry age and size effects on immersion immunization of brook trout, *Salvelinus-fontinalis* mitchell, against infectious pancreatic necrosis virus. *Journal of Fish Diseases* **13**, 113-125.

Bootland, L.M., Dobos, P. and Stevenson, R.M.W. (1995). Immunization of adult brook trout, *Salvelinus-fontinalis* (mitchill), fails to prevent the infectious pancreatic necrosis virus (IPNV) carrier state. *Journal of Fish Diseases,* **18**, 449-458.

Boudinot, P., Massin, P., Blanco, M., Riffault, S. and Benmansour, A., (1999). *vig-1*, a new fish gene induced by the rhabdovirus glycoprotein, has a virus-induced homolgogue in humans and shares conserved motifs with the MoaA family. *Journal of Virology*, **73**, 1846-1852.

Bowden, T.J., Smail, D.A. and Ellis, A.E. (2002). Development of a reproducible infectious pancreatic necrosis virus challenge model for Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases,* **25**, 555-563.

Braun-Nesje, R., Bertheussen, K., Kaplan, G. and Seljelid, R. (1981). Salmonid macrophages: separation, *in vitro* culture and characterization. *Journal of Fish Diseases*, **4**, 141-151.

Bricknell, I. and Dalmo, R.A. (2005). The use of immunostimulants in fish larval aquaculture. *Fish and Shellfish Immunology*, **19**, 457-472.

Bridle, A.R., Carter, C.G., Morrison, R.N., Nowak, B.F. (2005). The effect of *ß*-glucan administration on macrophage respiratory burst activity and Atlantic salmon, *Salmo salar* L., challenged with amoebic gill disease-evidence of inherent resistance. *Journal of Fish Diseases*, **28**, 347-356.

Bruslind, L.D. and Reno, P.W. (2000). Virulence comparison of three Buhl-Subtype isolates of infectious pancreatic necrosis virus in Brook trout fry, *Journal of Aquatic Animal Health*, **12**, 301-315.

Bustin, S.A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology*, **25**, 169-193.

Bustin, S.A. and Nolan, T. (2004). Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *Journal of Biomolecular Techniques*, **15**:155-166.

Bustin, S.A., Benes, V., Nolan, T. and Pfaffl, M.W. (2005). Quantitative real-time RT-PCR- a perspective. *Journal of Molecular Endocrinology*, **34,** 597-601**.**

Campagna, M., Eichwald, C., Vascotto, F. and Burrone, O.R. (2005). RNA interference of rotavirus segment 11 mRNA reveals the essential role of NSP5 in the virus replicative cycle, *Journal of General Virology*, **86**, 1481-1487.

Candan, A. (2002). First report on the diagnosis of infectious pancreatic necrosis (IPN) based on reverse transcription polymerase chain reaction (RTPCR) in Turkey. *Bulletin of the European Association of Fish Pathologists,* **22**, 45-48.

Cann, A.J. (2001). *Principles of molecular virology,* 3rd edn. San diego: Academic press.

Chen, D. and Ainsworth, A.J. (1992). Glucan administration potentiates immune defence mechanisms of channel catfish, *Ictalurus punctatus* Rafinesque. *Journal of Fish Diseases*, **15**, 295-304.

Chien, C-C., Bartel, P.L., Sternglanz, R. and Fields, S. (1991). The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest, *Proc. Natl. Acad. Sci*, **88**, 9578-9582.

Chou, H.Y., Peng, T.Y., Chang, S.J., Hsu, Y.L. and Wu, J.L. (1999). Effect of heavy metal stressors and salinity shock on the susceptibility of grouper (*Epinephelus sp.*) to infectious pancreatic necrosis virus. *Virus Research,* **63**, 121-129.

Christie, K.E (1997). Immunization with viral antigens: Infectious pancreatic necrosis. In Gudding, R., Lillehaug, A., Midtlyng, P.J. and Brown, F., (Eds.) *Fish vaccinology*, pp. 191-199. Karger.

Collet, B. and Secombes, C.J. (2001). The rainbow trout (*Oncorhynchus mykiss*) Mx1 promoter - Structural and functional characterization. *European Journal of Biochemistry,* **268**, 1577-1584.

Collet, B. and Secombes, C.J. (2002). Type I-Interferon signalling in fish, *Fish and Shellfish Immunology*, **12**, 389-397.

Collet, B., Boudinot, P., Benmansour, A., Secombes, C.J. (2004) An Mx1 promoter-reporter system to study interferon pathways in rainbow trout, *Developmental and Comparative Immunology*, **28**, 793-801.

Collet, B., Munro, E.S., Gahlawat, S., Acosta, F., Garcia, J., Roemelt, C., Zou, J., Secombes, C.J. and Ellis, A.E. (2007). Infectious pancreatic necrosis virus suppresses type I interferon signalling in rainbow trout gonad cell line but not in Atlantic salmon macrophages, *Fish and Shellfish Immunology*, **22**, 44-56.

Cutrin, J.M., Olveira, J.G., Barja, J.L. and Dopazo, C.P. (2000). Diversity of infectious pancreatic necrosis virus strains isolated from fish, shellfish, and other reservoirs in northwestern Spain. *Applied and Environmental Microbiology,* **66**, 839-843.

Cutrin, J.M., Lopez-Vazquez, C., Olveira, J.G., Castro, S., Dopazo, C.P. and Bandin, I. (2005). Isolation in cell culture and detection by PCR-based technology of IPNV-like virus from leukocytes of carrier turbot, *Scophthalmus maximus* (L.). *Journal of Fish Diseases*, **28**, 713-722.

Dalmo, R.A. and Seljeild, R. (1995). The immunomodulatory effect of LPS, laminaran and sulphated laminaran [*ß* (1,3)-D-glucan] on Atlantic salmon, *Salmo salar* L., macrophages *in vitro*. *Journal of Fish Diseases*, **18**, 175-185

Dalmo, R.A., Bøgwald, J., Ingebrigtsen, K. and Seljelid, R. (1996). The immunomodulatory effect of laminaran [*ß* (1,3)-D-glucan] on Atlantic salmon, *Salmo salar* L., anterior kidney leucocytes after intraperitoneal, peroral and peranal administration. *Journal of Fish Diseases*, **19**, 449-457.

Dalmo, R.A., Ingebrigtsen, K. and Bøgwald, J. (1997). Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). *Journal of Fish Diseases*, **20**, 241-273.

Damsgard, B., Mortensen, A. and Sommer, A.-I. (1998). Effects of infectious pancreatic necrosis virus (IPNV) on appetite and growth in Atlantic salmon, *Salmo salar* L (1998) *Aquaculture*, **163**, 185-193.

De Sena, J. and Rio, G.J. (1975). Partial purification and Characterization of RTG-2 fish cell Interferon, *Infection and Immunity*, **11**, 815-822.

Dobos, P., Hill, B.J., Hallett, R., Kells, D.T.C., Becht, H. and Teninges, D. (1979) Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. *Journal of Virology*, **32,** 593-605.

Dobos, P. (1995). The molecular biology of infectious pancreatic necrosis virus (IPNV). *Annual Review of Fish Diseases,* **5**, 25-54.

Dorson, M., Castric J. and Torchy, C. (1978). Infectious pancreatic necrosis virus and antigenic features of a pathogenic strain and of a non-pathogenic varient selected in RTG-2 cells. *Journal of Fish Diseases,* **1**, 309-320.

Dorson, M. (1988). Vaccination against infectious pancreatic necrosis. In: Ellis, A.E., (Ed.) *Fish Vaccination*, pp. 162-171. London: Academic Press.

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

Drolet, B.S., Chiou, P.P., Heidel, J. and Leong, J.A.C. (1995). Detection of truncated virus-particles in a persistent RNA virus-infection in-vivo. *Journal of Virology,* **69**, 2140-2147.

Du, G, Yonekubo, J, Zeng, Y, Osisami, M and Frohman, M.A. (2006). Design of expression vectors for RNA interference based on miRNAs and RNA splicing, *FEBS Journal*, **273**, 5421-5427.

Duncan, R., Nagy, E., Krell, P.J., Dobos, P. (1987). Synthesis of the infectious pancreatic necrosis virus polyprotein, detection of virus-encoded protease, and fine structure mapping of genome segment A coding regions. *Journal of Virology*, **61**, 3655-3664.

Eggset, G., Mortensen, A., Johansen, L.H. and Sommer, A.I. (1997). Susceptibility to furunculosis, cold water vibriosis, and infectious pancreatic necrosis (IPN) in post-smolt Atlantic salmon (*Salmo salar* L.) as a function of smolt status by seawater transfer. *Aquaculture,* **158**, 179-191.

Einer-Jensen, K., Bjorklund, H., Oreshkova, S., Shchelkunov, I., Vesely, T. and Lorenzen, N. (2002). Detection and typing of fish viruses. *Bulletin of the European Association of Fish Pathologists,* **22**, 158-165.

Ellermann-Eriksen, S. (2005) Macrophages and cytokines in the early in the early defence against herpes simplex virus, *Virology Journal*, **2**, 59.

Ellis, A.E. (1977). The leucocytes of fish: A review. *Journal of Fish Biology*, **11**, 453-491.

Ellis, A.E. (1988). *Fish vaccination*, London: Academic press.

Ellis, A.E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Developmental and Comparative Immunology,* **25**, 827-839.

Engstad, R.E. and Robertsen, B. (1993). Recognition of yeast cell wall glucan by Atlantic salmon (Salmo salar L.) macrophages. *Developmental and Comparative Immunology*, **17**, 319-330.

Espinoza, J.C. and Kuznar, J. (2002). Rapid simultaneous detection and quantitation of infectious pancreatic necrosis virus (IPNV). *Journal of Virological Methods,* **105**, 81-85.

Estepa, A. and Coll, J.M. (1991). Infection of mitogen-stimulated trout leukocytes with salmonid viruses. *Journal of Fish Diseases*, **14**, 555-562.

Ferguson, H.W. (1989). *Systematic pathology of fish: a text and atlas of comparitive tissue responses in diseases of teleosts*, Ames: Iowa State University press.

Ferko, B., Stasakova, J., Romanova, J., Kittel, C., Sereinig, S., Katinger, H. and Egorov, A. (2004). Immunogenicity and Protection Efficacy of Replication-Deficient Influenza A Viruses with Altered NS1 Genes, *Journal of Virology*, **78**, 13037-13045.

Fields, S. and Song, O.K. (1989). A novel genetic system to detect protein-protein interactions. *Nature*, **340**, 245-246.

Freeman, W.M., Walker, S.J. and Vrana, K.E. (1999). Quantitative RT-PCR: Pitfalls and Potential. *BioTechniques*, **26**, 112-125.

Frost, P. and Ness, A. (1997). Vaccination of Atlantic salmon with recombinant VP2 of infectious pancreatic necrosis virus (IPNV), added to a multivalent vaccine, suppresses viral replication following IPNV challenge. *Fish and Shellfish Immunology,* **7**, 609-619.

Frucht, D.M., Fukao, T., Bogdan, C., Schindler, H., O'Shea, J.J. and Koyasu, S. (2001). IFN-γ production by antigen-presenting cells: mechanisms emerge, *TRENDS in Immunology*, **22**, 556-560.

Garcia, J., Urquhart, K. and Ellis, A.E. (2006). Infectious pancreatic necrosis virus establishes an asymtomptomatic carrier state in kidney leucocytes of juvenile Atlantic cod, *Gadus morhua* L. *Journal of Fish Diseases*, **29**, 409-413.

Garcia-Sastre, A. (2002). Mechanisms of inhibition of the host interferon α/ß-mediated antiviral responses by viruses, *Microbes and Infection*, **4**, 647-655.

Gentle, A., Anastasopoulos, F. and McBrien, N.A. (2001). High-resolution semi-quantitative real-time PCR without the use of a standard curve. *BioTechniques*, **31**, 502-508.

Gessani, S. and Belardelli, F. (1998). IFN-γ Expression in Macrophages and its possible biological significance, *Cytokine and Growth Factors Reviews*, **9**, 117-123.

Ginzinger, D.G. (2002). Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Haematology*, **30**, 503-512.

Goodbourn, S., Didcock, L. and Randall, R.E. (2000). Interferons: cell signalling, immune modulation, antiviral responses and viral countermeasures. *Journal of General Virology*, **81**, 2341-2364.

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

Gratacap, R.M.L., Thompson, K.D., Bricknell, I.R. and Adams, A. (2006). Lipopolysaccharides extraction: Application of Butan-1-ol method to *Vibrio anguillarum.*

Fifth International Symposium on Aquatic Animal health. September 2-6, San Francisco, California.

Guselle, N.F., Markham, R.J.F. and Speare, D.J. (2006). Intraperitoneal administration of *β*-1,3/1,6-glucan to rainbow trout, *Oncorhynchus mykiss* (Walbaum), protects against *Loma salmonae*. *Journal of Fish Diseases*, **29**, 375-381.

Halder, M. and Ahne, W. (1988). Freshwater crayfish *Astacus astacus* - a vector for infectious pancreatic necrosis virus (IPNV). *Diseases of Aquatic Organisms,* **4**, 205-209.

Haller, O., Kochs, G. and Friedemann W., (2006). The interferon response circuit: Induction and suppression by pathogenic viruses. *Virology*, **344**, 119-130.

Havarstein, L.S., Endresen, C., Hjeltnes, B., Christie, K.E. and Glette, J. (1990). Specific immunoglobulins in serum from Atlantic salmon, *Salmo salar* L, immunized with vibrio-salmonicida and infectious pancreatic necrosis virus. *Journal of Fish Diseases,* **13**, 101-111.

Haynes, L.M., Moore, D.D., Kurt-Jones, E.A., Finberg, R.W., Anderson, L.J. and Tripp, R.A. (2001). Involvement of Toll-Like receptor 4 in Innate Immunity to Respiratory Syncytial Virus, *Journal of Virology*, **75**, 10730-10737.

Hedrick, R.P. and Fryer, J.L. (1982). Persistent infections of salmonid cell lines with infectious pancreatic necrosis virus (IPNV): A model for the carrier state in trout. *Fish Pathology,* **16**, 163-172.

Heppell, J. and Davis, H.L. (2000). Application of DNA vaccine technology to aquaculture. *Advanced Drug Delivery Reviews,* **43**, 29-43.

Hierholzer, J.C. and Killington, R.A. (1996). Virus isolation and quantification. In: Mahy, B.W.J. and Kangro, H.O., (Eds.). *Virology Methods Manual*. Great Britian, Academic Press Limited pp. 25-46.

Hill, B.J. (1982). Infectious pancreatic necrosis virus and its virulence. In: Roberts, R.J., (Ed.) *Microbial diseases of fish*, pp. 91-114. London: Academic Press

Hill, B.J. and Way, K. (1995). Serological classification of infectious pancreatic necrosis (IPN) virus and other aquatic birnaviruses. *Annual Review of Fish Diseases,* **5**, 55-77.

Honda, K., Yanai, H., Takaoka, A. and Taniguchi, T. (2005). Regulation of the Type I IFN induction: a current view. *International Immunology*, **17**, 1367-1378.

Hong, J.R., Lin, T.L., Hsu, Y.L. and Wu, J.L. (1998a). Apoptosis precedes necrosis of fish cell line with infectious pancreatic necrosis virus infection. *Virology,* **250**, 76-84.

Hong, J.R., Lin, T.L., Hsu, Y.L. and Wu, J.L. (1998b). Induction of apoptosis and secondary necrosis by infectious pancreatic necrosis virus in fish embryonic cells. *Journal of Fish Diseases,* **21**, 463-467.

Hong, J.R., Lin, T.L., Yang, J.Y., Hsu, Y.L. and Wu, J.L. (1999). Dynamics of nontypical apoptotic morphological changes visualized by green fluorescent protein in living cells with infectious pancreatic necrosis virus infection. *Journal of Virology,* **73**, 5056-5063.

Hong, J.R., Gong, H.Y. and Wu, J.L. (2002). IPNV VP5, a novel anti-apoptosis gene of the Bcl-2 family, regulates Mcl-1 and viral protein expression. *Virology,* **295**, 217-229.

Hoshino, K., Kaisho, T., Iwabe, T., Takeuchi, O. and Akira, S. (2002). Differential involvement of IFN-ß in Toll-like receptor-stimulated dendritic cell activation. *International Immunology*, **14**, 1225-1231.

Jarp, J., Gjevre, A.G., Olsen, A.B. and Bruheim, T. (1995). Risk-factors for furunculosis, infectious pancreatic necrosis and mortality in post-smolt of Atlantic salmon, *Salmo-salar* L. *Journal of Fish Diseases,* **18**, 67-78.

Jarp, J., Taksdal, T. and Torud, B. (1996). Infectious pancreatic necrosis in Atlantic salmon *Salmo salar* in relation to specific antibodies, smoltification, and infection with erythrocytic inclusion body syndrome (EIBS). *Diseases of Aquatic Organisms,* **27**, 81-88.

Jashes, M., Gonzalez, M., Lopezlastra, M., Declercq, E. and Sandino, A. (1996). Inhibitors of infectious pancreatic necrosis virus (IPNV) replication. *Antiviral Research,* **29**, 309-312.

Jensen, I., and Robertsen B. (2000). Cloning of an Mx cDNA from Atlantic halibut (Hippolossus hippoglossus) and characterisation of Mx mRNA expression in response to double-stranded RNA of infectious pancreatic necrosis virus. *Journal of Interferon and Cytokine Research*, **20**, 701-710.

Jensen, I., Larsen, R. and Robertsen, B., (2002). An antiviral state induced in Chinhook salmon embryo cells (CHSE-214) by transfection with the double-stranded RNA poly I:C. *Fish and Shellfish Immunology*, **13**, 367-378.

Jensen, I. and Robertsen, B. (2002). Effect of double-stranded RNA and interferon on the antiviral activity of Atlantic salmon cells against infectious salmon anemia virus and infectious pancreatic necrosis virus. *Fish and Shellfish Immunology,* **13**, 221-241.

Jensen, I., Albuquerque, A., Sommer, A-I. and Robertsen, B. (2002). Effect of poly I:C on the expression of Mx proteins and resistance against infection by infectious salmon anaemia virus in Atlantic salmon. *Fish and Shellfish Immunology*, **13**, 311-326.

Johansen, L.H. and Sommer, A.I. (1995) Multiplication of infectious pancreatic necrosis virus (IPNV) in head kidney and blood leucocytes isolated from Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases*, **18**, 147-156.

Johansen, L.H. and Sommer, A.I. (2001) Infectious pancreatic necrosis virus infection in Atlantic salmon *Salmo salar* post-smolts affects the outcome of secondary infections with infectious salmon anaemia virus or *Vibrio salmonicida*. *Diseases of Aquatic Organisms,* **47**, 109-117.

Johansen, A., Collet, B., Sandaker, E., Secombes, C.J. and Jørgensen, J.B. (2004). Quantification of Atlantic salmon type-I interferon using an Mx promoter reporter gene assay. *Fish and Shellfish Immunology*, **16**, 173-184.

Joost Haasnoot, P.C., Cupac, D. and Berkhout, B. (2003). Inhibition of Virus Replication by RNA Interference, *Journal of Biomedical Science,* **10**, 607-616.

Jørgensen, J.B. and Robertsen, B. (1995). Yeast *ß*-glucan stimulates respiratory burst activity of Atlantic salmon (Salmo salar L.) macrophages. *Developmental and Comparative Immunology*, **19**, 43-57.

Jørgensen, J.B., Johansen, A., Stenersen, B. and Sommer, A.I. (2001). CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (Salmo salar L.) leucocytes to produce supernatants with antiviral activity. *Developmental and Comparitive Immunology*, **25**, 313-321.

Jørgensen, J.B., Johansen, A., Sterio, K. and Johansen, A. (2003). CpG DNA induces protective antiviral immune responses in Atlantic salmon (*Salmo salar* L.). *Journal of Virology*, **77**, 11471-11479.

Jørgensen, S.M., Lyng-Syvertsen, B., Lukacs, M., Grimholt, U. and Gjøen, T (2006). Expression of MHC class I pathway genes in response to infectious salmon anaemia virus in Atlantic salmon (Salmon salar L.) cells. *Fish and Shellfish Immunology*, **21**, 548-560.

Kapadia, S.B., Brideau-Anderson, A. and Chisari, F.V. (2003). Interference of hepatitis C virus RNA replication by short interfering RNAs, *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 2014-2018.

Kato, A., Homma, T., Batchelor, J., Hashimoto, N., Imai, S., Wakiguchi, H., Saito, H. and Matsumoto, K. (2003). Interferon-alpha/beta receptor-mediated selective induction of a gene cluster by CpG oligodeoxynucleotide 2006, *BMC Immunology,***4**, 1-10

Kaufmann, S.H.E. (1993). Immunity to intracellular bacteria. *Annual Review of Immunology,* **11**, 129-163.

Kent, M.L. and Poppe, T.T. (1998). *Diseases of seawater netpen-reared salmonid fishes*, Nanaimo, BC, Canada: Quadra Printers Ltd.

Kerr, M.K., Martin, M., and Churchill, G.A. (2000). Analysis of variance for gene expression microarray data, *Journal of Computational Biology,* **7**, 819–839.

Kim, C.H., Dummer, D.M., Chiou, P.P. and Leong, J.A.C. (1999). Truncated particles produced in fish surviving infectious hematopoietic necrosis virus infection: mediators of persistence? *Journal of Virology,* **73**, 843-849.

Kim, C.H., Johnson, M.C., Drennan, J.D., Simon, B.E., Thomann, E. and Leong, J.A.C. (2000) DNA vaccines encoding viral glycoproteins induce nonspecific immunity and mx protein synthesis in fish. *Journal of Virology,* **74**, 7048-7054.

Knott, R.M., Munro, A.L. (1986). The persistence of infectious pancreatic necrosis virus in Atlantic salmon. *Veterinary Immunology and Immunopathology*, **12**, 359-364.

Krönke, J., Kittler, R., Buchholz, F., Windisch, M.P., Pietschmann, T., Bartenschlager, R. and Frese, M. (2004). Alternative Approaches for Efficient Inhibition of Hepatitis C Virus RNA Replication by Small Intefering RNAs, *Journal of Virology*, **78**, 3436-3446.

Kumari, J. and Sahoo, P.K. (2006). Dietary *ß*-1,3 glucan potentiates innate immunity and disease resistance of Asian catfish, *Clarias batrachus* (L.). *Journal of Fish Diseases*, **29**, 95-101.

Kuo, C.C., Kuo, C.W., Liang, C.M. and Liang, S.M. (2005) A transcriptomic and proteomic analysis of the effect of CpG-ODN on human THP-1 monocytic leukemia cells, *Proteomics* **5**, 894–906.

Kutyavin, I.V., Afonina, I.A., Mills, A., Gorn, V.V. Lukhtanov, E.A. Belousov, E.S., Singer, M.J., Walburger, D.K., Lokhov, S.G., Gall, A.A., Dempcy, R., Reed, M.W., Meyer, R.B., & Hedgpeth, J. (2000). 3'-minor groove binder–DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Research,* **28,** 655–661.

Labus, M.B., Breeman, S., Ellis, A.E., Smail, D.A., Kervick, M. and Melvin, W.T. (2001). Antigenic comparison of a truncated form of VP2 of infectious pancreatic necrosis (IPN) virus expressed in four different cell types. *Fish and Shellfish Immunology,* **11**, 203-216.

Laing, K.J. and Secombes, C.J. (2004). Chemokines, *Developmental and Comparative Immunology,* **28**, 443-460.

Lancaster, M.U., Hodgetts, S.I., Mackenzie, J.S. and Urosevic, N. (1998). Characterization of defective viral RNA produced during persistent infection of vero cells with Murray Valley encephalitis virus. *Journal of Virology,* **72**, 2474-2482.

Larsen, R., Røkenes, T. and Robertsen, B. (2004). Inhibition of Infectious Pancreatic Necrosis Virus Replication by Atlantic salmon Mx1 Protein, *Journal of Virology*, **78**, 7938-7944.

Lee, J.Y., Hirono, I. and Aoki, T. (2000). Cloning and analysis of expression of Mx cDNA in Japanese flounder, *Paralichthys olivaceus*. *Developmental and Comparative Immunology,* **24**, 407-415.

Leong, J.A.C., Trobridge, G.D., Kim, C.H.Y., Johnson, M. and Simon, B. (1998). Interferon-inducible Mx proteins in fish. *Immunological Reviews,* **166**, 349-363.

Levy, D.E. & Garcia-Sastre, A. (2001). The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. *Cytokine & Growth Factor Reviews*, **12**, 143-156.

Livak, K. (1997). ABI Prism 7700 Sequence Detection System, User Bulletin 2. PE Applied Biosystems, Foster City, CA.

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods*, **25**, 402-408.

Lockhart, K., McBeath, A.J.A., Collet, B. Snow, M and Ellis, A.E. (2006). Expression of Mx mRNA following infection with IPNV is greater in IPN-susceptible Atlantic salmon post-smolts than in IPN-resistant Atlantic salmon parr, *Fish and Shellfish Immunology*, **22**,151-156.

Long, S., Wilson, M., Bengten, E., Bryan, L., Clem, W., Miller, N.W. and Chinchar, V.G. (2004). Identification of a cDNA encoding channel catfish interferon. *Developmental Comparative Immunology*, **28**, 97-111.

Lopezlastra, M., Gonzalez, M., Jashes, M. and Sandino, A.M. (1994). A detection method for infectious pancreatic necrosis virus (IPNV) based on reverse transcription (RT)-polymerase chain- reaction (PCR). *Journal of Fish Diseases,* **17**, 269-282.

Lui, W. and Saint, D.A. (2002). A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. *Analytical Biochemistry*, **302,** 52-59.

Machida, K., Cheng, K.T.H., Sung, V.M.-H., Levine, A.M., Foung, S. and Lai, M.M.C. (2006). Hepatitus C Virus induces Toll-like Receptor 4 Expression, leading to enhanced production of Beta Interferon and Interleukin-6, *Journal of Virology*, **80**, 866-874.

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish and Shellfish Immunology* **20**, 137-151.

Magyar, G., Chung, H.K. and Dobbs, P. (1998). Conversion of VP1 to VPg in cells infected by infectious pancreatic necrosis virus. *Virology,* **245**, 142-150.

Manning, M.J. and Nakanashi, T. (1996). The Specific Immune System: Cellular defenses. In: *The Fish Immune System: Organism, Pathogen, and Environment*. (Iwama, G., Nakanishi, T. ed) pp.159-205. San Diego: Academic Press.

Marié, I., Durbin, J.E. and Levy, D.E. (1998). Differential viral induction of distinct interferon-α genes by positive feedback through interferon regulatory factor-7, *The EMBO Journal*, **17**, 6660-6669.

Matsumoto, M., Kikkawa, S., Kohase, M., Miyake, K. and Seya, T. (2002). Establishment of a monoclonal antibody against human Toll-receptor 3 that blocks double-stranded RNA-mediated signalling. *Biochemical and Biophysical Research Communications*, **293**, 1364-1369.

Mcallister, P.E. and Owens, W.J. (1992) Recovery of infectious pancreatic necrosis virus from the feces of wild piscivorous birds. *Aquaculture,* **106**, 227-232.

Mcallister, P.E. and Bebak, J. (1997). Infectious pancreatic necrosis virus in the environment: Relationship to effluent from aquaculture facilities. *Journal of Fish Diseases,* **20**, 201-207.

McBeath, A.J.A., Snow, M., Secombes, C.J., Ellis, A.E. and Collet, B. (2007). Expression kinetics of interferon and interferon-induced genes in Atlantic salmon (*Salmo salar*)

following infection with infectious pancreatic necrosis virus and infectious salmon anaemia virus. *Fish and Shellfish Immunology*, **22**, 230-241.

Melby, H.P., Caswell-Reno, P. and Falk, K. (1994). Antigenic analysis of Norwegian aquatic Birnavirus isolates using monoclonal antibodies with special reference to fish species, age and health status. *Journal of Fish Diseases*, **17**, 85-91.

Melroe, G.T., Deluca, N.A. and Knipe, D.M. (2004). Herpes simplex virus 1 has multiple mechanisms for blocking virus-induced interferon production. *Journal of Virology*, **78**, 8411-8420.

Midtlyng, P.J., Storset, A., Michel, C., Slierendrecht, W.J. and Okamoto, N. (2002). Breeding for disease resistance in fish. *Bulletin of the European Association of Fish Pathologists,* **22**, 166-172.

Milne, S.A., Gallacher, S., Cash, P. and Porter, A.J.R. (2006). A reliable RT-PCR-ELISA method for the detection of infectious pancreatic necrosis virus (IPNV) in farmed rainbow trout. *Journal of Virological Methods,* **132,** 92-96.

Moody, C.E., Serreze, D.V. and Reno, P.W. (1985). Non-specific cyto-toxic activity of teleost leukocytes. *Developmental and Comparative Immunology,* **9**, 51-64.

Mortensen, S.H. (1993). Passage of infectious pancreatic necrosis virus (IPNV) through invertebrates in an aquatic food-chain. *Diseases of Aquatic Organisms,* **16**, 41-45.

Moya, J., Pizarro, H., Jashes, M., De Clercq, E. and Sandino, A.M. (2000). In vivo effect of EICAR (5-ethynyl-1-beta-D- ribofuranosylimidazole-carboxamide) on experimental infected rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*Onchorhynchus kisutch*) fry with infectious pancreatic necrosis virus. *Antiviral Research,* **48**, 125-130.

Mulero, V. and Mesenguer, J. (1998). Functional characterisation of a macrophage-activiating factor produced by leucocytes of gilthead seabream (*Sparus aurata* L.) *Fish and Shellfish Immunology,* **8**, 143-156.

Muller, P.Y., Janovjak, A., Miserez, A.R. and Dobbie, Z. (2002). Processing of gene expression data generated by quantitative real-time RT-PCR. *BioTechniques*, **32**, 1372-1379.

Muñoz-Jordán, J.L., Sánchez-Burgos, G.G., Laurent-Rolle, M. and Garcia-Sastre, A. (2003). Inhibition of interferon signalling by dengue virus, *Microbiology*, **24**, 14333-14338.

Munro, E.S., Gahlawat, S.K. and Ellis, A.E. (2004). A sensitive non-destructive method for detecting IPNV carrier Atlantic salmon, *Salmo salar* L., by culture of virus from plastic adherent blood leucocytes. *Journal of Fish Diseases*, **27**, 129-134.

Munro E.S., Gahlawat, S.K., Acosta, F. and Ellis A.E. (2006). In Infectious pancreatic necrosis virus carrier Atlantic salmon, Salmo salar L., post smolts, almost all kidney macrophages ex vivo contain a low level of non-replicating virus. *Journal of Fish Diseases*, **29**, 43-46.

Munzo, J., Esteban, M.A. and Meseguer, J. (1999). *In vitro* culture requirements of sea bass (*Dicentrarchus labrax* L.) blood cells: Differential adhesion and phase contrast microscopic study. *Fish and Shellfish Immunology*, **9**, 417-428.

Murray, A.G., Busby, C.D. and Bruno, D.W. (2003) Infectious pancreatic necrosis virus in Scottish Atlantic salmon farms, 1996-2001. *Emerging Infectious Diseases,* **9**, 455-460.

Murray, A.G. (2006 a). A model of the emergence of infectious pancreatic necrosis virus in Scottish salmon farms 1996-2003. *Ecological Modelling*, **199**, 64-72.

Murray, A.G. (2006 b). Persistence of infectious pancreatic necrosis virus (IPNV) in Scottish salmon (*Salmo salar* L.) farms. *Preventive Veterinary Medicine*, **76**, 97-108.

Nakanishi, T., Fischer, U., Dijkstra, J.M., Hasegawa, S., Somamoto, T., Okamoto, N. and Ototake, M. (2002). Cytotoxic T cell function in fish. *Developmental and Comparative Immunology,* **26**, 131-139.

Neumann, N.F., Fagan, D. and Belosevic, M. (1995). Macrophage activating factor(s) secreted by mitogen stimulated goldfish kidney leukocytes synergize with bacterial lipopolysaccharide to induce nitric oxide production in teleost macrophages. *Developmental and Comparative Immunology*, **19**, 473-482.

Noga, E.J. (2000) *Fish disease: diagnosis and treatment*, Ames, IA: Iowa state university press.

Novoa, B., Barja, J.L. and Figueras, A. (1995) Entry and sequential distribution of an aquatic birnavirus in turbot (*Scophthalmus-maximus*). *Aquaculture,* **131**, 1-9.

Nova, B., Figueras, A., Ashton, I. and Secombes, C.J. (1996). In vitro studies on the regulation of rainbow trout (Oncorhynchus mykiss) macrophage respiratory burst activity. *Developmental and Comparative Immunology*, **20**, 207-216.

Nova, B., Figueras, A., Ashton, I. and Secombes, C.J. (1996 b). Effects of in vitro addition of infectious pancreatic necrosis virus (IPNV) on rainbow trout *Oncorhynchus mykiss* leucocyte responses. *Veterinary Immunology and Immunopathology*, **51**, 365-376.

Nygaard, R., Husgard, S., Sommer, A.I., Leong, J.A.C. and Robertsen, B. (2000). Induction of Mx protein by interferon and double-stranded RNA in salmonid cells. *Fish & Shellfish Immunology* **10**, 435-450.

Nyman, T.A., Matikainen, S., Sareneva, T., Julkunen, I., and Kalkkinen, N. (2000). Proteome analysis reveals ubiquitin-conjugating enzymes to be a new family of interferon-α-regulated genes. *European Journal of Biochemistry*, **267**, 4011-4019.

O'Farrell, C., Vaghefi, N., Contonnet, M., Buteau, B., Boudinot, P. and Benmansour, A. (2002). Survey of transcript expression in rainbow trout leukocytes reveals a major contribution of interferon-responsive genes in the early response to a rhabdovirus infection. *Journal of Virology*, **76**, 8040-8049.

Okamoto, N., Shirakura, T., Nagakura, Y. and Sano, T. (1983). The mechanism of interference with fish viral infection in the RTG-2 cell line. *Fish Pathology,* **18**, 7-12.

Oldstone, M.B.A. (2006). Viral persistence: Parameters, mechanisms and future predictions. *Virology*, **344**, 111-118.

Oye, A.K. and Rimstad, E. (2001). Inactivation of infectious salmon anaemia virus, viral haemorrhagic septicaemia virus and infectious pancreatic necrosis virus in water using UVC irradiation. *Diseases of Aquatic Organisms,* **48**, 1-5.

Park, J.W. and Jeong, G.J. (1996). Identification of VP3 as an important neutralising epitope from DRT strain, a Korean isolate of infectious pancreatic necrosis virus (IPNV). *Fish & Shellfish Immunology,* **6**, 207-219.

Paulsen, S.M., Lunde, H., Engstad, R.E. and Robertsen, B. (2003). In vivo effects of *ß*-glucan and LPS on regulation of lysozyme activity and mRNA expression in Atlantic salmon (*Salmo salar* L.). *Fish and Shellfish Immunology*, **14**, 39-54.

Peat, S., Whelan, W.J. and Lawley, H.G. (1958). The structure of laminarin. Part I. The main polymeric linkage. *Journal of the Chemical Society*, 724-728.

Peters, I.R., Helps, C.R., Hall, E.J. and Day, M.J. (2004). Real-time RT-PCR: considerations for efficient and sensitive assay design. *Journal of Immunological* Methods, **286**, 203-217.

Pettersen, E.F., Ulvenes, M., Melingem, G.O. and Wergeland, H.I. (2003). Peripheral blood and head kidney leucocyte populations during out-of-season (0+) parr-smolt transformation and seawater transfer of Atlantic salmon (*Salmo salar* L.) *Fish and Shellfish Immunology*, **15**, 373-385.

Pettersen, E.F., Bjørløw, I., Hagland, T.J. and Wergeland, H.I. (2005). Effect of seawater temperature on leucocyte populations in Atlantic salmon post-smolts. *Vet. Immunological Immounopathology,* **15**, 65-76

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, **29**, 2002-2007.

Pfaffl, M.W., Horgan, G.W. and Dempfle, L. (2002) Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research*, **30**, 1-10.

Pierson, S.N., Butler, J.N. and Foster, R.G. (2003). Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. Nucleic Acids Research, **31**, e73.

Plouffe, D.A, Hanington, P.C., Walsh, J.C., Wilson, E.C. and Belosevic, M. (2005). Comparison of select innate immune mechanisms of fish and mammals. *Xenotransplantation*, **12**, 266-277.

Post, G. (1987). *Textbook of fish health*, 2nd edn. Ascot: T.F.H. Publications.

Rae, G. (2002). Sea lice control is Scotland, past and present. *Pest Management Science*, **58**, 515-520.

Randonic, A., Thulke, S., Mackay, I.M., Landt, O., Siegert, W. and Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and Biophysical Research Communications*, **313**, 856-862.

Reno, P.W. (1999). Infectious pancreatic necrosis and associated aquatic birnaviruses. In: Woo, P.T.K. and Bruno, D.W., (Eds.) *Fish diseases and disorders: volume 3: viral, bacterial and fungal infection,* pp. 1-55. Oxon: CAB International

Rise, M.L., Jones, S.R.M., Brown, G.D., Von Schalburg, K.R., Davidson, W.S. and Koop, B.F. (2004). Microarray analyses identify molecular biomarkers of Atlantic salmon macrophage and haematopoietic kidney response to *Piscirickettsia salmonis* infection. *Pysiol Genomics,* **20**, 21-35.

Robersten, B. (2006). The interferon system of teleost fish. *Fish & Shellfish Immunology*, **20**,172-191.

Roberts, R.J. (1989). *Fish pathology,* 2nd edn. London: Baillere tindall.

Roberts, R.J. and Shepherd, C.J. (1997). *Handbook of trout and salmon diseases*, 3rd edn. Farnham: Fishing news books.

Robertsen, B., Trobridge, G. and Leong, J.A. (1997). Molecular cloning of double-stranded RNA inducible MX genes from Atlantic salmon (*Salmo salar* L.). *Developmental and Comparative Immunology* **21**, 397-412.

Robertsen, B., Rorstad, G., Engstad, R. and Raa, J. (1990). Enhancement of non-specific disease resistance in Atlantic salmon, *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls. *Journal of Fish diseases*, **13**, 391-400.

Robertsen, B. (1999). Modulation of the non-specific defence of fish by structurally conserved microbial polymers. *Fish and Shellfish Immunology*, **9**, 269-290.

Robertsen, B., Bergen, V., Røkenes, T., Larsen, R. and Albuquerque, A. (2003). Atlantic salmon interferon genes: cloning, sequence analysis, expression and biological activity. *Journal of Interferon Cytokine Research*, **23**, 601-612.

Rønneseth, A., Pettersen, E.F. and Wergeland, H.I. (2006). Neutrophils and B-cells in blood and head kidney of Atlantic salmon (*Salmo salar* L.) challenged with infectious pancreatic necrosis virus (IPNV). *Fish and Shellfish Immunology*, **20**, 610-620.

Sadasiv, E.C. (1995). Immunological and pathological responses of salmonids to infectious pancreatic necrosis virus (IPNV). *Annual Review of Fish Diseases* **5**, 209-223.

Saint-Jean, S.R. and Pérez-Prietom, S.I. (2006). Interferon mediated antiviral activity against salmonid fish viruses in BF-2 and other cell lines, *Veterinary Immunology and Immunopathology*, **110**, 1-10.

Sakai, M. (1999). Current research of fish immunostimulants. *Aquaculture*, **172**, 63-92.

Salinas, I., Lockhart, K., Bowden, T.J., Collet, B, Secombes, C.J. and Ellis, A.E. (2004). An assessment of immunostimulants as Mx inducers in Atlantic salmon (*Salmo salar* L.) parr and the effect of temperature on the kinetics of Mx responses, *Fish & Shellfish Immunology,* **17**, 159-170.

Samuel, C.E. (2001). Antiviral Actions of Interferons. *Clinical Microbiology Reviews*, **14**, 778-809.

Sangrador-Vegas, A., Martin, S.A.M., O'Dea, P.G. and Smith, T.J. (2000). Cloning and characterization of the Rainbow trout (Oncorhynchus mykiss) type II interleukin-1 receptor cDNA. *European Journal of Biochemistry*, **267**: 7031-7037.

Santi, N., Vakharia, V.N., and Evensen Ø. (2004) Identification of putative motifs involved in the virulence of infectious pancreatic necrosis virus. *Virology*, **322**, 31-40.

Santi, N., Song, H., Vakharia, V.N. and Evensen, Ø. (2005). Infectious pancreatic necrosis virus VP5 is dispensable for virulence and persistence. *Journal of Virology*, **79**, 9206-9216.

Sarmento, A., Marques, F., Ellis, A.E. and Afonso, A. (2004). Modulation of the activity of sea bass (*Dicentrarchus labrax*) head-kidney macrophages by macrophage activating factor(s) and lipopolysaccharide. *Fish and Shellfish Immunology*, **16**, 79-92.

Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N. and Taniguchi, T. (2000). Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction, *Immunity*, **13**, 539-548.

Savan, M. and Dobos, P. (1980). Effect of virazole on rainbow trout *Salmo gairdneri* richardson fry infected with infectious pancreatic necrosis virus. *Journal of Fish Diseases,* **3**, 437-440.

Schmitz, F., Mages, J., Heit, A., Lang, R., and Wagner, H. (2004). Transcriptional activation induced in macrophages by Toll-like receptor (TLR) ligands: from expression profiling to a model of TLR signaling, *European Journal of Immunology,* **34**, 2863–2873.

Secombes, C.J. (1990). Isolation of salmonid macrophages and analysis of their killing activity. In: *Techniques in Fish Immunology* (ed. By J.S. Stolen, D.P. Fletcher, B.S. Anderson & W.B. van Muiswinkel), pp.137-154. SOS Publications, Fair Haven, NJ, USA.

Secombes, C.J. (1994). The phylogeny of cytokines. In: *The Cytokine Handbook*, 2nd edition (A.W. Thompson, ed.) pp.567-594. London: Academic Press.

Sen, G.C. (2001). Viruses and Interferons, *Annual review of microbiology*, **55**, 255-281.

Shioda, T., Andriole, S., Yahata, T. and Isselbacher, K.J. (2000). A green fluorescent protein-reporter mammalian two-hybrid system with extrachromosomal maintenance of a prey expression plasmid:Application to interaction screening. Proceedings of the National *Academy of Sciences of the United States of America*, **97**, 5220-5224.

Shivappa, R.B., Song, H., Yao, K., Aas-Eng, A., Evensen, Ø. and Vakharia, V.N. (2004). Molecular characterization of Sp serotype strains of infectious pancreatic necrosis virus exhibiting differences in virulence. *Diseases of Aquatic Organisms*, **61**, 23-32.

Smail, D.A., Bruno, D.W., Dear, G., McFarlane, L.A. and Ross, K. (1992). Infectious pancreatic necrosis (IPN) virus Sp serotype in farmed Atlantic salmon, *Salmo salar* L., post-smolts associated with mortality and clinical diseases. *Journal of Fish Diseases,* **15**, 77-83.

Smail, D.A., Huntly, P.J. and Munro, A.L.S. (1993a). Fate of 4 fish pathogens after exposure to fish silage containing fish farm mortalities and conditions for the inactivation of infectious pancreatic necrosis virus. *Aquaculture,* **113**, 173-181.

Smail, D.A., Irwin, N., Harrison, D. and Munro, A.L.S. (1993b). Passage and survival of infectious pancreatic necrosis (IPN) virus in the cows gut after feeding a silage mixture containing IPN virus. *Aquaculture,* **113**, 183-187.

Smail, D.A., Mcfarlane, L., Bruno, D.W. and Mcvicar, A.H. (1995). The pathology of an IPN-Sp sub-type (Sh) in farmed Atlantic salmon, *Salmo salar* L, post-smolts in the Shetland Isles, Scotland. *Journal of Fish Diseases,* **18**, 631-638.

Solem, S.T., Jørgensen, J.B. and Robertsen, B. (1995). Stimulation of respiratory burst and phagocytic activity in Atlantic salmon (*Salmo salar* L.) macrophages by lipopolysaccharide. *Fish and Shellfish Immunology*, **5**, 475-491.

Somamoto, T., Nakanishi, T. and Okamoto, N. (2000). Specific cell-mediated cytotoxicity against a virus-infected syngeneic cell line in isogeneic ginbuna crucian carp. *Developmental and Comparative Immunology,* **24**, 633-640.

Song, H., Santi, N., Evensen, Ø. and Vakharia, V.N. (2005). Molecular determinants of infectious pancreatic necrosis virus virulence and cell culture adaptation. *Journal of Virology*, **79**, 10289-10299.

Sørensen, K K., Sveinbjørnsson, B., Dalmo, R A., Smedsrød, B., and Bertheussen, K. (1997). Isolation, cultivation and characterization of head kidney macrophages from Atlantic cod, *Gadus morhua* L, *Journal of Fish Diseases*, **20**, 93-107.

Steuerwald, N., Cohen, J., Herrera, R.J. and Brenner, C.A. (1999). Analysis of gene expression in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR. *Molecular Human Reproduction*, **5**, 1034-1039.

Stoskopf, M.K. (1993). *Fish medicine*, Philadelphia: W.B. Saunders Company.

Swanson, R.N. and Gillespie, J.H. (1981). An indirect fluorescent-antibody test for the rapid detection of infectious pancreatic necrosis virus in tissues. *Journal of Fish Diseases,* **4**, 309-315.

Swanson, R.N. and Gillespie, J.H. (1982). Isolation of infectious pancreatic necrosis virus from the blood and blood components of experimentally infected trout. *Canadian Journal of Fisheries and Aquatic Sciences,* **39**, 225-228.

Taffala, C., Nova, B. (2000) Requirements for nitric oxide production by turbot (*Scophthalmus maximus)* head kidney macrophages. *Developmental and Comparative Immunology*, **24,** 623-631.

Tafalla, C., Coll, J. and Secombes, C.J. (2005). Expression of genes related to the early immune response in rainbow trout (*Oncorhynchus mykiss*) after viral haemorrhagic septicaemia virus (VHSV) infection. *Developmental Comparative Immunology*, **29**, 615-626.

Taksdal, T., Poppe, T., Sivertsen, T. and Ferguson, H.W. (1995). Low-levels of vitamin-e in plasma from atlantic salmon *Salmo- salar* with acute infectious pancreatic necrosis (IPN). *Diseases of Aquatic Organisms,* **22**, 33-37.

Taksdal, T., Ramstad, A., Stangeland, K. and Dannevig, B.H. (1998). Induction of infectious pancreatic necrosis (IPN) in covertly infected Atlantic salmon, *Salmo salar* L., post smolts by stress exposure, by injection of IPN virus (IPNV) and by cohabitation. *Journal of Fish Diseases,* **21**, 193-204.

Taksdal, D. (1999). Infectious pancreatic necrosis (IPN) in Atlantic salmon: Infection trials, pathogenesis and diagnostic methods. PhD thesis. Norwegian college of veterinary medicine. Oslo, ISBN 82-90550-3.

Taksdal, T., Dannevig, B.H. and Rimstad, E. (2001). Detection of infectious pancreatic necrosis (IPN)-virus in experimentally infected Atlantic salmon parr by RT-PCR and cell culture isolation. *Bulletin of the European Association of Fish Pathologists,* **21**, 214-219.

Tan, Y-D., Fornage, M. and Fu, Y.U. (2006). Ranking analysis of microarray data: A powerful method for identifying differentially expressed genes. *Genomics*, **88**, 846-854.

Taniguchi, T. and Takaoka, A. (2002). The interferon- α/ß system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors, *Current Opinion in Immunology*, **14**, 111-116.

Tosi, M.F. (2005). Innate immune responses to infection, *Journal of Allergy and Clinical Immunology*, **116**, 241-249.

Trobridge, G.D., Chiou, P.P. and Leong, J.A.C. (1997). Cloning of the rainbow trout (*Oncorhynchus mykiss*) Mx2 and Mx3 cDNAs and characterization of trout Mx protein expression in salmon cells. *Journal of Virology,* **71**, 5304-5311.

Trobridge, G.D., Chiou, P.P., Kim, C.H. and Leong, J.A.C. (1997). Induction of the Mx protein of rainbow trout *Oncorhynchus mykiss in vitro* and *in vivo* with poly I:C dsRNA and infectious hematopoietic necrosis virus. *Diseases of Aquatic Organisms,* **30**, 91-98

Ullu, E., Djikeng, A., Shi, H. and Tschudi, C. (2002). RNAi interference: advances and questions. *Philosophical transactions of the Royal society B: Biological sciences*, **357**, 65-70.

Valarcher, J-F., Furze, J., Wyld, S., Cook, R., Conzelmann, K-K. and Taylor, G. (2003). Role of Alpha/Beta Interferons in the Attenuation and Immunogenicity of Recombinant Bovine Respiratory Syncytical Viruses Lacking NS Proteins. *Journal of Virology*, **77**, 8426-8349.

Wang, A.M., Doyle, M.V. and Mark, D.F. (1989). Quantitation of mRNA by the polymerase chain reaction. *Proceedings of the National Academy of Sciences*, **86**, 9717-9721.

Wang, Z., Ren, L., Zhao, X., Hung, T., Meng, A., Wang, J. and Chen, Y-G. (2004). Inhibition of severe acute respiratory syndrome virus replication by small interfering RNAs in mammalian cells, *Journal of Virology*, **78**, 7523-7527.

Weber, S., Fichtner, D., Mettenleiter, T.C. and Mundt, E. (2001). Expression of VP5 of infectious pancreatic necrosis virus strain VR299 is initiated at the second in-frame start codon. *Journal of General Virology,* **82**, 805-812.

Wergeland, H.I. and Aakre Johansen, R. (2001). A salmonid cell line (TO) for production of infectious salmon anaemia virus (ISAV). *Diseases of Aquatic Organisms*, **44**, 183-190.

Williams, K., Blake, S., Sweeney, A., Singer J. T., and Nicholson B. L. (1999). Multiplex Reverse Transcriptase PCR Assay for Simultaneous Detection of Three Fish Viruses. *Journal of Clinical Microbiology*, **37**, 4139-4141.

Wolf, K. (1988). *Fish viruses and fish viral diseases*, 1st edition. New York: Cornell University press.

Wong, M.L. and Mendrano, J.F. (2005). Real-time PCR for mRNA quantitation. *Biotechniques*, **39**, 1-11.

Yabu, T., Hirose, H., Hirono, I., Katagiri, T., Aoki, T. and Yamamoto, E. (1998). Molecular cloning of a novel interferon regulatory factor in Japanese flounder, *Paralichthys olivaceus*, *Molecular Marine Biology and Biotechnology*, **7**, 138-144.

Yamamoto, T. (1975). Infectious pancreatic necrosis (IPN) virus carriers and antibody production in a population of rainbow trout (*salmo gairdneri*). *Candian Journal of Microbiology,* **21**, 1343-1347.

Yoon, J-B., Kim, S-H., Shin, M-C., Lee, D-G., Hong, S-K., Jung, Y-T., Khang, I-G., Shin, W-S, Kim, C-C. and Paik, S-Y. (2004). Inhibition of Herpesvirus-6B RNA Replication by Short Interference RNAs, *Journal of Biochemistry and Molecular Biology*, **37**, 383-385.

Yoshiura, Y., Kiryu, I., Fujiwara, A., Suetake, H., Suzuki, Y., Nakanishi, T. and Ototake, M. (2003). Identification and characterization of Fugu orthologues of mammalian interleukin-12 subunits. *Immunogenetics*, **55**, 296-306.

Yu, K. K-Y., MacDonald, R.D. and Moore, A.R. (1982). Replication of infectious pancreatic necrosis virus in trout leucocytes and detection of the carrier state. *Journal of Fish Diseases*, **5**, 401-410.

Zhang, Y.B., Hu C.Y., Zhang J., Huang G.P., Wei L.H., Zhang, Q.Y. and Gui, J.F. (2003). Molecular cloning and characterization of crucian carp (*Carassius auratus L.*) interferon regulatory factor 7, *Fish and Shellfish Immunology*, **15**, 453-466.

Zhang, Z., Ding, L. and Sandford, A.J. (2005). Selection of reference genes for gene expression studies in human neutrophils by real-time PCR. *BCM Molecular Biology*, **6**, 1-7.

Zou, J., Carrington, A., Collet, B., Dijkstra, J.M., Yoshiura, Y., Bols, N. and Secombes, C. (2005). Identification and Bioactivities of IFN-γ in Rainbow Trout *Oncorhynchus mykiss*: The First Th1-Type Cytokine Characterized Functionally in Fish, *The Journal of Immunology*, **175**, 2484-2494.

Zou, J., Wang, T., Hirono I., Aoki, T., Inagawa, H. and Honda, T. (2002). Differential Expression of two tumor necrosis factor genes in rainbow trout, *Oncorhynchus mykiss*, *Developmental and Comparative Immunology*, **26**, 161-172.