AN ABSTRACT OF THE THESIS OF

Linda D. Bruslind for the degree of Doctor of Philosophy in Microbiology presented on January 7, 1997. Title: Pathogenic and Molecular Characterization of Three Closely Related Isolates of Infectious Pancreatic Necrosis Virus (IPNV).

Abstract approved: ——

Paul W. Reno

Three closely related isolates belonging to the A_1 serotype of infectious pancreatic necrosis virus (IPNV) were selected for comparison, to provide insight into the nature of variation in the virulence of IPN viruses. Brook trout fry (*Salvelinus fontinalis)* were experimentally infected with the three isolates by immersion. Cumulative mortalities over a 62 day period for the three isolates were 67%, 78%, and 93%. The negative control was 3%. Virus titers from whole fish homogenates sampled at peak mortality for each isolate were statistically similar, indicating that quantity of virus does not account for virulence differences. For the two least virulent isolates, the virus titer was inversely correlated with fish weight, whereas for the most virulent isolate, no correlation was observed.

Amino acid sequences of the viral capsid protein VP2 were determined using the reverse transcriptase polymerase chain reaction (RT-PCR). There were two amino acid changes at residue 217 and 288 between the two least virulent isolates and the most virulent isolate. These changes might provide a specific molecular basis for the variations in virulence among isolates.

The progression of IPN virus infection in the experimentally infected fry was followed using histopathology, *in situ* cDNA hybridization, and alkaline phosphatase immunohistochemistry (APIH). While microscopic lesions were limited almost exclusively to necrosis of the pyloric caeca and pancreas, positive reactions with *in situ* hybridization and APIH were observed in tissues throughout infected fish. An IPNV infection appeared

to be established in the fish by two routes: by entering the skin/lateral line and diffusing through the muscle, and from the oral region into the gastrointestinal tract by ingestion.

In a second experiment, within a group of experimentally infected brook trout fry, external and behavioral signs of IPN disease in moribund fish disappeared, with the fish becoming healthy in appearance. Several of these fish were sampled, along with dead, moribund, and asymptomatic fish (never showed signs of IPN disease). Very few differences were observed among the fish sampled, using histopathology and *in situ* hybridization. Fish that appeared to recover after displaying signs of IPN disease died within a 2 week period.

Pathogenic and Molecular Characterization of Three Closely Related Isolates of Infectious Pancreatic Necrosis Virus (IPNV)

by

Linda D. Bruslind

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Linda D. Bruslind, Author

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CHAPTER 1
THESIS INTRODUCTION

Infectious pancreatic necrosis virus (IPNV) is a virus which infects aquatic animals that can cause devastating disease in salmonid species worldwide. It is known to cause significant losses in hatchery-reared brook and rainbow trout (*Salvelinus fontinalis* and *Oncorhynchus mykiss*) in both eastern and western parts of the U.S., and throughout the world. It has caused notable mortality in young salmonids in Oregon hatcheries (Groberg *et al.*, 1980), and is considered a prevailing fish health problem in Idaho fish hatcheries (Hauck, 1990).

Two recent occurrences in the U.S. succinctly illustrate the concern raised by the IPN virus. In one instance, 7 million young salmonids from a single hatchery facility in Colorado were destroyed to eliminate IPNV (P. Reno, personal communication). In the other instance, IPNV was detected in returning coho salmon (*Oncorhynchus kisutch*), resulting in the destruction of more than 2 million eggs from this stock of fish (Olson et al., 1994). In Europe, an increased association of IPNV with postsmolt disease of farmed Atlantic salmon (*Salmo salar*) has been observed. In the two months following transfer of smolts to sea, poor growth and significant mortality occur that can be related to pancreas and gastrointestinal pathologies associated with high IPN virus titers (Smail *et al.*, 1992). In Norway, losses up to 30% have been reported for IPN outbreaks in postsmolts (Christie *et al.*, 1990).

The unenveloped, icosahedral virus is about 60 nm in size and has been classified as a birnavirus, with a genome consisting of two segments of double-stranded RNA (Dobos, 1976). The virus is transmitted both horizontally and vertically, with survivors from IPNV enzootics becoming carriers for life (Bootland *et al.*, 1991). The virus has been detected in both freshwater and marine fish from 32 different families, as well as

members of the mollusc and crustacean phyla (Hill and Way, 1995); it is most often detected in carrier animals in the absence of overt disease. Given the worldwide distribution of the virus in freshwater and marine environments, combined with the frequent lack of external disease signs, the potential for dissemination of the IPN virus is very high.

IPNV is one of the most extensively studied fish viruses, and much has been discovered about the molecular biology of IPNV, including its mode of replication and the genomic segments responsible for production of each viral polypeptide product (Dobos and Roberts, 1983; Dobos, 1995). However, little is known about the pathogenesis of the disease. A large part of the difficulty in understanding the disease is a lack of information about why overt disease occurs in certain situations and not in others. Therefore, it is important to determine which inherent virus characteristics are associated with the virulence of IPN virus.

Ten different birnavirus serotypes exist, within two serogroups designated A and B (Hill and Way, 1995). All IPN viruses are members of Serogroup A, which contains 9 different serotypes. Serotypes are differentiated by reciprocal cross-neutralization assays, using standardized procedures. Each serotype is composed of numerous isolates, with the virulence expressed by different IPNV isolates being extremely variable (Jorgensen and Kehlet, 1971; Sano, 1971b). Recent sequence data (B. Nicholson, personal communication) from the VP2 viral capsid protein region believed to be responsible for virulence (Sano *et al.*, 1991) indicates an extreme homology among IPNV isolates. These data belie marked differences in virulence among the isolates, based on mortality experiments using susceptible brook trout fry (J. Maret, personal communication). The purpose of this research was to focus on several isolates of IPNV with marked variations in virulence, but virtually identical sequences, to determine how the isolates differ in mechanisms of infection.

Chapter 3 of this thesis describes a possible molecular basis for the difference in virulence among Pacific Northwest strains of IPNV in salmonids, by determining the nucleotide sequence of three isolates of Buhl subtype IPNV that appear to differ markedly in their expressed virulence. Susceptible brook trout fry were experimentally infected with

the different isolates of IPNV, and the resulting mortality data used to confirm the virulence level of each isolate. The viral genome was extracted and sequenced, using the reverse-transcriptase polymerase chain reaction (RT-PCR), at three different points in time: before being introduced into the fish, during the epizootic, and 2 months after exposure, in order to determine if major changes occurred in the VP2 region that might account for the differences in virulence at a molecular level.

Chapter 4 of this thesis describes research that utilized both molecular probes and immunohistochemical techniques in tandem with conventional histopathology to determine the pathogenesis of the IPN disease in brook trout fry. Resulting reactions in individual tissues of the fish were also compared among isolates of different virulence levels to determine if differing mechanisms of infection could be elucidated, or whether there was a predilection for certain tissues by more virulent isolates, which was lacking in less virulent isolates.

Lastly, in Chapter 5, this thesis examines the possible survival of fish infected with IPNV that display clinical signs of IPN disease, in order to ascertain information about why some fish survive an IPNV infection while others die. It has been presumed that fish exhibiting signs of disease die within hours of the onset of signs (Wolf, 1966).

CHAPTER 2
LITERATURE REVIEW

Infectious Pancreatic Necrosis Disease

History

Infectious pancreatic necrosis disease (IPN) is believed to have been first described by M'Gonigle in 1941, who observed whirling behavior and high mortality in brook trout (*Salvelinus fontinalis*) fry in Canada. At the time, however, the condition was described as "acute catarrhal enteritis" and the likely cause was thought to be nutritional or physiological.

After a similar outbreak in the eastern U.S. in 1954, the infectious nature of IPN was subsequently demonstrated by Wood *et al.* (1955), using waterborne transmission to healthy brook trout. Infection resulted in acute pancreatic necrosis in young fry. Based on the results of these experiments, a virus was suggested by Wood *et al.* to be the causative agent of IPN.

Snieszko *et al.* (1957, 1959) reported possible outbreaks of the IPN disease in rainbow trout (*Onchorynchus mykiss*), Atlantic salmon (*Salmo salar*), and brown trout (*Salvelinus trutta*) fingerlings; and subsequently demonstrated the transmissibility of IPN by feeding a homogenate of moribund IPN infected fry to susceptible brook trout fingerlings.

The actual viral nature of IPN was proven in 1960 by Wolf *et al.*, who infected cell cultures of eastern brook trout tissues, using gill, swim bladder, spleen, kidney, and caudal fin of infected fish. Cytopathic effect (CPE) was exhibited by the cell cultures in 18-24 h after primary inoculation, with complete destruction of the cells occurring in 1-4 days. It was also found at this time that the agent was filterable, unaffected by antibiotics, and could be passed on serially in cell culture. When susceptible fish were inoculated with the cell culture fluid, IPN signs developed (Wolf *et al.*, 1961), thus fulfilling Rivers' postulates (Rivers, 1937) and proving this virus to be the etiologic agent of the disease.

Host Range

Although IPN is most prominently known for its devastating effects on freshwater salmonids, such as brook trout, rainbow trout, brown trout, and cutthroat trout (*Oncorhynchus clarki*), the virus has been found in numerous other aquacultured and feral species, both freshwater and marine.

The first non-salmonid host to be discovered carrying the IPN virus was a population of healthy white suckers (*Catastomus commersoni*) located downstream from an IPN-positive Canadian fish hatchery (Sonstegard *et al.*, 1972). Since that time, the list of affected species has grown to include fish that represent 32 different families, as well as members of the mollusc and crustacean phyla (Hill and Way, 1995). In most of these cases, the virus was found without clinical signs of disease being present in the host species. However, there is convincing evidence that IPNV does cause disease in some non-salmonid hosts, such as young Japanese eels (*Anguilla japonica*) (Sano *et al.*, 1981), as well as yellowtail (*Seriola quinqueradiata*) fry and fingerlings in Japan (Sorimachi and Hara, 1985).

It was suggested that IPNV causes disease in Atlantic menhaden (*Brevoortia tyrannus*), after a virus similar to IPNV was isolated from Atlantic menhaden suffering from a "spinning" disease in Chesapeake Bay (Stephens *et al.*, 1980). Susceptible fish that were experimentally infected with the isolated virus exhibited hemorrhaging at the base of fins and along the body, along with darkened pigmentation, followed by erratic swimming behavior and death within 3-5 days. The virus could be re-isolated from the brain, pancreas and other internal organs, as well as the blood. Similar viruses were found in striped bass (*Morone saxatilis*) (Schultz *et al.*, 1984), and Mediterranean sea bass (*Dicentrarchus labrax*) (Bonami *et al.*, 1983), although it has not been conclusively decided whether the disease signs caused by these virions meet the criterion for IPN disease.

To date, IPN virus has not been found infecting a mammalian species. Indeed, no pathogenic mammalian birnaviruses have been discovered (Dobos, 1995).

Epidemiology

Clinical signs of IPN disease are displayed primarily by early feeding fry or fingerlings, with an almost linear relationship reported between the decrease in sensitivity to the virus with increasing age of the susceptible host (Dorson and Torchy, 1981). Mortalities resulting from IPNV infection have traditionally been recorded in hatchery fish less than 6 months of age. However, IPN disease of yearling salmonids has been recorded in brook trout (Elazhary *et al.*, 1976) and experimentally infected Atlantic salmon (Swanson and Gillespie, 1979), and recent observations at European sea farm sites indicate that IPNV is causing increased disease and mortality in postsmolt Atlantic salmon (Krogsrud *et al.*, 1989; Christie and Havarstein, 1989; Christie *et al.*, 1990; Smail *et al.*, 1992). Additionally, it has been observed that asymptomatic carriers of IPNV who survived an outbreak of the disease as fry may have a reactivation of clinical disease, mediated by stress (Roberts and McKnight, 1976). It does appear that a susceptible fish of any age can become infected with the IPN virus, subsequently becoming an asymptomatic, chronic carrier.

Although the time course of the disease varies considerably with factors such as host species and age, water temperature, and virus strain, some general observations can be made from experimental evidence. Clinical signs of the disease usually appear in feeding fry about 3-5 days after initial exposure to the virus, while fingerlings manifest signs about 8-10 days after exposure (Noga, 1996). Peak mortality usually occurs from 7 to 18 days after exposure, after which mortalities taper off relatively quickly (Swanson and Gillespie, 1979; Lapeirre *et al.*, 1988). A second, smaller peak of mortalities occurring a few days after the first may or may not be observed (P. Reno, personal communication). Overall, mortalities from IPNV usually last a total of 4-6 weeks after initial exposure to the virus, although fish that have been weakened by the virus may sporadically die over a longer period of time.

Environmental Factors

As with most fish diseases, water temperature is one of the most important environmental factors affecting the outcome of an IPN infection. Generally, at high temperatures (10° to 14° C), mortality of infected fry is the most rapid and extreme. However, at temperatures greater than 15°C, the mortality rate is suppressed (Dorson and Torchy, 1981). At lower temperatures, mortality of the infected fish becomes prolonged and the mortality rate is often reduced, although susceptibility to IPNV infection at specific temperatures appears to depend primarily upon the virus isolate (Frantsi and Savan, 1971).

There is also evidence that environmental conditions such as overcrowding affect the development of clinical IPN in hatchery-reared fry or fingerlings. In the field, it has been observed that the mortality level caused by IPN becomes lower if the population density of hatchery-reared fish is reduced (Jorgensen and Bregnballe, 1969). Other stress inducers such as transportation and low oxygen content have been observed to cause increased replication of the IPN virus in carrier fish (Frantsi and Savan, 1971; Roberts and McKnight, 1976), and it has been suggested that stress-promoting treatments such as immunosuppressant reagents might be used to increase the titer of IPNV in order to aid detection capabilities (McAllister *et al.*, 1994).

Pathology

The characteristic signs of IPN disease include corkscrew whirling about the long axis alternating with prostration, ascites which causes severe distension of the abdomen, petechial hemorrhages of ventral areas, castlike excretions from the vent, overall darkening, and rapid respiration. Exopthalmia may or may not be present. Infected fish refuse feed and collect at the water outflow. Following the most severe signs, death is often rapid, usually occurring within an hour or two (Wolf, 1966).

Internally, the spleen, heart, kidneys and liver of infected fish are pale, and there is petechial hemorrhage in the pyloric caeca and adipose tissue. The digestive tract is devoid of food, replaced by copious amounts of a clear to milky mucous in the stomach and anterior intestine. Large amounts of sloughed off epithelial cells from the pyloric caeca and intestinal mucosa are also found throughout the digestive tract.

Fry that survive an acute IPN infection often become significantly stunted in their growth because of the extensive pancreatic fibrosis that may occur (McKnight and Roberts, 1976; Munro and Duncan, 1977).

Histopathology

As is evident from the name, pronounced pancreatic inflammation and necrosis are the most obvious signs of disease seen in tissue sections of infected fish. The pancreatic acinar cells are the prime viral target, although islet pancreatic tissues may be affected, as well as the adjacent adipose tissue. Lymphocytes usually infiltrate into the acinar tissue in the early stages of infection, followed by nuclear pyknosis and karyorhexis in acinar cells (Noga, 1996). The epithelial cells of the pyloric caeca may swell and develop a fragmented nucleus, becoming "McKnight cells", which then shed their eosinophilic cytoplasm into the lumen of the pyloric caeca (McKnight and Roberts, 1976).

Sloughing of the mucosal epithelium is common in the pylorus, pyloric caeca, and upper intestine. The liver, kidney, and hematopoietic tissue may show slight pathological signs, such as focal necrosis or increased macrophages (Yasutake *et al.*, 1965; Swanson and Gillespie, 1979).

Hyaline degeneration of the skeletal muscle of infected fish is frequently described in association with IPN disease (Snieszko *et al.*, 1957), although a direct correlation has not been proven (Wolf, 1966). Acinar cells may contain basophilic, cytoplasmic inclusions, believed to be the products of cell degeneration, especially near the edges of tissue where necrosis has just begun (Noga, 1996). However, these signs are not consistent enough to be definitely associated with the disease.

Distribution

After the initial reports of IPN in Canada by M'Gonigle (1941) and in the eastern U.S. by Wood *et al.* (1955), IPN was also found to exist in the western U.S. (Parisot *et al.*, 1963). It was found to be widespread throughout fish culture stations in eastern Canada (MacKelvie and Artsob, 1969). It was quickly discovered to be present in many other areas worldwide.

The first European outbreak was reported affecting rainbow trout fry in France in 1964 (Besse and de Kinkelin, 1965), which was followed by a similar report of IPN disease signs manifested by rainbow trout in Denmark in 1968 (Jorgensen and Bregnballe, 1969). Sano (1971a) described the first outbreak in Asia, which occurred in rainbow trout fry in Japan. In further research, Sano (1972) discovered that IPN disease was widespread in Japanese rainbow trout fish hatcheries.

Subsequently, IPN disease occurrences have been reported worldwide, with the most frequent occurrences in major trout-producing areas of the United States, Canada, France, Denmark, Norway, and Japan. With the recent report describing isolation of IPNV from two fish hatcheries in Portugal (Sousa *et al.*, 1996), Greece remains the only member of the European Community (EC) to date that has not found IPNV in its facilities (Hill, 1992).

Natural Transmission

The transmission of the highly contagious IPN virus is both horizontal and vertical. Natural horizontal transmission occurs in the water from carrier fish to susceptible fry, and from infected fry to susceptible fry, via waterflow, contact, or cannibalism of infected tissue. Virus may be shed in the urine, feces, and sex products of infected fish, although not all infected fish shed virus and some may shed only intermittently (Billi and Wolf, 1969). One major source of IPN virus from infected fish may be the fecal pseudocast, which is highly mucoid. Additionally, IPNV was found to be present in the seminal fluid

of rainbow trout by Ahne (1983), while Mulcahy and Pascho (1984) found virus absorbed onto rainbow trout sperm.

Transmission occurs vertically from adult carriers to the progeny fry in or on the eggs and ovarian fluid (Wolf *et al.*, 1963; Bullock *et al.*, 1976). Ahne and Negele (1985) conducted several experiments involving both eyed eggs and sexual products of salmonids and found that the virus, which had a strong affinity for the egg shell, could be reisolated from egg shells more than 3 weeks after infection. Since yolk-sac fry were observed trying to eat the egg shells, it is possible that infected egg shells serve as a source of IPNV contamination for young fry. It has been suggested by Wolf (1988) that IPNV may contribute to the mortality of fish embryos.

Survivors from an enzootic infection become IPN virus carriers for life (Bootland *et al.*, 1991), serving as the reservoir of the infection. The virus has most often been detected in carrier animals in the absence of overt clinical disease.

Experimental Transmission

The disease can be transmitted experimentally by feeding infected material (Wolf *et al.*, 1961), injecting infected material intra-peritoneally or IP (Wolf, 1966), or by immersing susceptible fish in water containing the virus (Wood *et al.*, 1955; McAllister and Owens, 1986). Transmission of IPNV by immersion occurs either by contact through the water or by ingestion of the virus, leading to administration via the oral route.

Carriers/Vectors

Several non-salmonid aquaculture species have been implicated as possible transmission vectors or carriers for the IPN virus. The striped bass has been shown to spread the virus to brook trout (Wechsler *et al.*, 1987; McAllister and McAllister, 1988). Freshwater crayfish (*Astacus astacus*) infected with IPNV were found to excrete the virus

into the water continuously and the virus was found in the organs of infected animals for up to 1 year after initial infection (Halder and Ahne, 1988). Scallops (*Pecten maximus*) which had been artificially infected with IPNV by injection or immersion were found to shed the virus in their feces, while prawns (*Pandulus borealis* and *Palaemon elegons*) which fed on dead IPNV-contaminated scallops became carriers of the virus (Mortensen, 1993). Spawning prawns (*Panaeus japonicus*) from farms in Italy were also found to carry the IPN virus (Giorgetti, 1990).

In the 1980s, loons (*Gavia immer*) were observed to feed on IPN infected brook trout from ponds located in New York state (Flick, 1983). Although no evidence was obtained that the loons were spreading IPN virus to nearby ponds, it was suggested that predators might be a possible source of transmission. In 1992, IPNV was recovered from the feces of wild piscivourous birds that were observed feeding on IPN infected fish (McAllister and Owens, 1992). However, no disease signs were evident in the birds, and it remains unknown if they can serve as a vector for the virus.

Detection

The presence of clinical IPN in fish is usually determined by observation of classical pathological signs in susceptible species. However, in the absence of overt disease, clinical IPN can be detected by the inoculation of susceptible cell cultures with an overlay of filtered tissue homogenates derived from fish suspected to be infected with the virus. Target tissues for isolation of high titers include the posterior kidney, pancreas, pyloric caeca, spleen and liver. Typical CPE usually occurs in the inoculated cell culture within 2-4 days, depending on cell type, incubation temperature, and to a large extent, the amount of virus used to inoculate the culture.

A definitive diagnosis of IPNV infection can be obtained by combining cell line replication with one of several serological assays available, such as: the serum neutralization assay, direct or indirect immunofluorescence (Piper *et al.*, 1973; Nicholson and Henchal, 1978), the complement fixation test (Finlay and Hill, 1975), the enzyme-

linked immunosorbent assay or ELISA (Nicholson and Caswell, 1982; Dixon and Hill, 1983; Hattori *et al.*, 1984), or an enzyme immunodot (Caswell-Reno *et al.*, 1989). In all of these tests, IPNV specific antibodies (either mono- or polyclonal) are used to bind to the IPN virus in cell culture, if present, coupled with one of a variety of biochemical reactions for detection, providing a means for identification. For certain assays, it has been shown to be possible to test tissue homogenates from infected fish directly, without growth of the virus in cell culture, as long as the background absorbance is not prohibitively high (Dixon and Hill, 1983) and the virus titers are high ($\geq 10^6$/g).

Oligonucleotide DNA probes approximately 24 bases in size have also been used for the diagnosis of IPNV infection (Christie *et al.*, 1988; Rimstad *et al.*, 1990; Blake *et al.*, 1995). With these, it was found that an initial extraction of the IPNV RNA was not required. Cloned cDNA probes were developed by Dopazo *et al.* (1994) for use in a dot-blot hybridization assay to detect the presence of IPNV RNA in tissues of infected brook and rainbow trout. However, the assay was found to be most effective when combined with the conventional diagnostic method of inoculating the infected fish tissue homogenates onto a susceptible cell culture for 12-24 h before performing the assay, in order to increase the amount of viral RNA present.

Infectious Pancreatic Necrosis Virus

Virion Characteristics

Infectious pancreatic necrosis virus (IPNV) is the prototype virus of the Birnaviridae family, which was established relatively recently (Brown, 1986) and contains three genera. IPNV is the type species of the genus Aquabirnavirus, the designation indicating that it infects aquatic species. The virion, with a molecular weight of 55 x 10^6 Daltons or Da (Dobos *et al.*, 1977), is non-enveloped with an icosahedral capsid approximately 60 nm in diameter. Typically, the viral capsid has been described as being composed of 92 capsomers with a triangulation number (T) of 9 (Kelly and Loh, 1972;

Cohen *et al.*, 1973), however, it has been proposed that the capsid layer actually contains 132 capsomers with a T = 13 (Ozel and Gelderblom, 1985). The buoyant density of IPNV in CsCl is 1.33 g/cm^3 (Kelly and Loh, 1972).

Although originally believed to have a single-stranded RNA genome (Nicholson, 1971; Kelly and Loh, 1972), it was ultimately determined that each IPN virion contains a double-stranded RNA genome that is bipartite, consisting of two segments of RNase-resistant RNA. Neither segment contains a poly A tail at the 3' end. The segments are similar in size, with molecular weights of 2.5 x 10^6 and 2.3 x 10^6 Da (Dobos, 1976). The two genome segments, referred to as A and B, are functionally and structurally different, although they both contain large areas of noncoding sequence with extensive homology between the two segments. In contrast, there is little genomic homology between the coding regions of the two segments.

Proteins

Segment A, the larger RNA segment with 3097 bp, is bicistronic, containing two overlapping open reading frames (ORFs). A large ORF encodes three induced viral proteins, designated as VP2, VP3, and NS, after post-translational cleavage of a 106 kDa polyprotein (NH_2-preVP2-NS protease-VP3-COOH). A small ORF, in a different reading frame that overlaps the large ORF, encodes a 17 kDa minor polypeptide that is arginine-rich (Duncan *et al.*, 1987). After expressing the 17kDa polypepetide as a fusion protein, it was determined that this basic protein is synthesized in small quantities in infected fish cells. It was also found that at least one strain of IPNV lacked the small ORF, while two other strains had truncated ORFs (Heppell *et al.*, 1995).

VP2 is originally produced as preVP2 along with VP3, during cotranslational cleavage of the polyprotein by the viral protease, NS. The preVP2 is then further cleaved to yield the structural VP2 protein (Dobos and Rowe, 1977; Duncan *et al.*, 1987), which is 54 kDa in size. VP2 is one of the two capsid proteins of IPNV, representing 62% of the virion protein by mass. An external protein, VP2 stimulates production of type-specific

neutralizing monoclonal antibodies or MAbs (Nicholson, 1993), and it is believed by some to contain all the epitopes recognized by neutralizing MAbs (Caswell-Reno *et al.*, 1986; Tarrab *et al.*, 1995).

The other viral capsid protein, VP3, is 31 kDa in size and represents 28% of the virion protein by mass, although it is present in slightly larger quantities than VP2 (Dobos, 1995). Some IPNV serotypes contain a second small polypeptide that is 29 kDa in size, VP3a (sometimes referred to as VP4 in the literature), that is probably cleaved from VP3 during virus maturation (Dobos and Rowe, 1977). VP3 is believed by some researchers to be an internal protein (Dobos *et al.*, 1977; Dobos, 1977), although it has been suggested that at least a portion of the protein is exposed on the surface of the virion, based on results using anti-VP3 MAbs in both ELISA and immunodot assays (Caswell-Reno *et al.*, 1986; Caswell-Reno *et al.*, 1989; Nicholson, 1993). VP3 is known to contain several nonneutralizing epitopes based on the production of VP3 specific MAbs to several IPNV serotypes (Caswell-Reno *et al.*, 1986; Wolski *et al.*, 1986; Christie *et al.*, 1990; Dominguez *et al.*, 1991; Lecomte *et al.*, 1992; Tarrab *et al.*, 1996), and a recent publication described virus neutralizing activity using mouse antiserum raised against VP3/VP3a purified from the DRT strain of IPNV (Park and Jeong, 1996).

The non-structural protease protein, NS, is 28kDa in size and found only in trace amounts (Dobos and Rowe, 1977).

Segment B, the smaller RNA segment with 2784 bp, is monocistronic, encoding for the putative RNA-dependent RNA polymerase (RdRp), known as VP1 (Duncan *et al.*, 1991). VP1, which is 94 kDa in size, is an internal polypeptide found as two forms in the virion: as a free polypeptide, and as a genome-linked form known as VPg. In the latter form, a serine residue in the VPg forms a phosphodiester bond with the 5' of each RNA strand (Calvert *et al.*, 1991). Although the IPNV RdRp was found by Poch *et al.* (1989) to contain several conserved domains in common with RdRps from other RNA viruses, it is unique in that it is lacking a characteristic Gly-Asp-Asp motif usually associated with this enzyme family (Gorbalenya and Koonin, 1988; Duncan *et al.*, 1991).

Replication

From the results of binding experiments, using CHSE cells at 4°C, it has been determined that IPNV saturates cellular binding sites in 2-3 hours (Dobos, 1995). Competition experiments, again using CHSE-214 cells, have demonstrated that IPNV attaches to cells by means of specific cellular components (Kuznar *et al.*, 1995). The mechanisms for penetration or uncoating of the virus are presently unknown, although it was shown that a very small amount of the adsorbed IPNV actually enters the cells, becoming internalized in acid compartments within 20 minutes. Maximum synthesis of the viral RNA genome occurs 8-10 hours after initial infection of a cell, then diminishes by 14 hours post-infection (Somogyi and Dobos, 1980). Replication occurs in a semi-conservative manner, with displacement of the plus strand of genomic RNA by the newly synthesized RNA strand.

Transcription of the dsRNA genome is primed by the VP1 protein (Vpg), after it forms a phosphodiester bond with the 5' of each RNA strand. Synthesis of the viral proteins, VP1, preVP2, VP3 and NS, occurs in approximately equal amounts between 3-14 hours post-infection (Dobos, 1977).

Stability

The IPN virus is very stable in water, remaining infectious for up to 6 months in 10°C tap water (Toranzo and Hetrick, 1982). It gradually becomes inactivated, but its level of stability is about the same in fresh versus salt water, indicating the potential of IPNV to survive in a variety of aquatic environments (Desautels and MacKelvie, 1975; Toranzo and Hetrick, 1982). The temperature of the water, regardless of water type, greatly affects the rate of viral inactivation. At 15°C, IPNV is more stable in estuarine water, while at 20°C, its rate of inactivation is about the same in both estuarine and fresh water (Toranzo and Hetrick, 1982).

IPNV is sensitive to alkaline pH (12.2), but relatively resistant to acid pH (2.5), underlining the ability of the virus to endure the conditions of the fish gut (Vestergaard-Jorgensen, 1974). The virus is also resistant to air drying (Toranzo and Hetrick, 1982). It is relatively heat stable (MacKelvie and Desautels, 1975), and variably resistant to freezing, surviving for several months in frozen viscera (Noga, 1996). The virus can be successfully stored for several years by freezing at -80°C.

In fresh water or phosphate buffered saline (PBS), IPNV is inactivated by at least 25 ppm of iodophor or chlorine (Desautels and MacKelvie, 1975), however, the activity of these disinfectants greatly decreases in the presence of organic matter or serum in the environment (Inouye *et al.*, 1990). Efficacy of these disinfectants also depends upon the water pH and the concentration of the virus present (Elliott and Amend, 1978).

Additional substances that inactivate IPNV include cresol, ethanol, methanol, and formalin (Dixon and Hill, 1983), as well as treatment by ozonation. IPNV is moderately resistant to UV irradiation (Liltved *et al.*, 1995), and is not found to be inactivated by ether, propanol, phenol, or chloroform treatments (Inouye *et al.*, 1990).

Cell Culture

IPNV was first isolated in a primary fish cell culture from eastern brook trout in 1960 by Wolf *et al.* Since then it has been found to replicate well in the cell lines of many teleost fish species (Wolf and Mann, 1980), such as chinook salmon *Oncorhynchus tshawytscha* (Walbaum) embryo CHSE-214 cells, carp *epithelioma papulosum cyprini* EPC cells, rainbow trout *Oncorhynchus mykiss* gonad RTG-2 cells, and fathead minnow *Pimephales promelas* FHM cells. Research by Kelly *et al.* (1978) found that the RTG-2 cell line was more sensitive than the FHM cell line to lytic infection by IPNV. However, IPNV induces interferon production in RTG-2 cells, which have a sensitivity to interferon treatment, subsequently resulting in lower infectious titers around 10^6-10^7 PFU/ml (Macdonald and Kennedy, 1979) than in other cells. Since CHSE-214 cells are not sensitive to interferon treatment and are not producers of interferon, they are commonly

used by most laboratories to grow high titers of IPNV (Dobos, 1995). The titer of the virus in culture fluid from infected CHSE-214 cells is generally 10^8-10^9 PFU/ml or higher. Bovo *et al.* (1985), after comparing the sensitivity of five fish cell lines to IPNV, concluded that pike (*Esox lucius*) gonad PG cells (Ahne, 1979) were the best cells for detection and titration of IPNV, however, CHSE-214 cells were not included in the comparison.

The optimum temperature for propagation of IPNV in cell lines is 20°C, with a range of 4-27°C. This low temperature range is a viral requirement, as opposed to being a requirement of the host cells (Dobos, 1995).

A single cycle of viral replication takes 16-20 h at 22°C, with IPNV infected cell lines producing a characteristic CPE in 2-3 days. Many of the cells become spindle-shaped, remaining attached to the substrate surface, with nuclear pyknosis evident. Other cells round up and separate from neighboring cells in the monolayer. Eventually, as the infection progresses, the majority of cells detach from the substrate surface and lyse, resulting in almost total cell destruction. Some cells might stick to the substrate, appearing to be alive.

Avian and mammalian cell lines are not susceptible to infection with IPNV, possibly due to the low temperature requirement and/or the lack of necessary viral attachment receptors. Likewise, insect cell lines are not susceptible to infection with IPNV, although they can grow at the lower temperatures (Dobos, 1995). This suggests the absence of a receptor for IPNV.

Types

The original prototype isolate of IPNV was isolated by Wolf *et al.* (1960) from brook trout undergoing an epizootic in West Virginia, and deposited in the American Type Culture Collection (ATCC) in 1963. The designation "VR-299" refers to the reference number given to the isolate. The VR-299 antiserum was used extensively to test new found virus isolates, to determine if they were IPNV strains, based on neutralization

assays. However, many researchers (Malsberger and Cerini, 1963; MacKelvie and Artsob, 1969; Wolf *et al.*, 1969) found that there was wide variation in the level of neutralization observed, using the VR-299 antiserum. It was suggested that IPNV had greater antigenic diversity than previously recognized and that different serotypes of IPNV might exist. This led to new classifications of the IPN viruses.

Three well-documented serotypes of IPNV were originally described in the literature, referred to as Sp (Jorgensen and Bregnballe, 1969), Ab (Jorgensen and Grauballe, 1971), and VR-299 (Wolf *et al.*, 1960). They can be differentiated by neutralization tests as well as by physical and phenotypic differences (MacDonald and Gover, 1981). Sp and Ab were isolated from Denmark, while VR-299 was found in the U.S. Most U.S. strains of IPNV are related to VR-299, the original reference strain, while Sp and Ab are defined as European isolates.

A proposal by B.J. Hill (1982) divided the aquatic birnaviruses into two serogroups: Serogroup I which contains all IPN viruses, and Serogroup II, an unrelated group which includes *Tellina* virus I (TV-I) as the type isolate.

After an extensive study comparing 175 IPN virus isolates from eleven countries using polyclonal antisera in a neutralization assay, a further proposal by Hill & Way (1983) divided Serogroup I into nine cross-reacting serotypes, referred to as West Buxton (WB), Sp, Ab, Hecht (He), Tellina (Te), Canada 1 (C1), Canada 2 (C2), Canada 3 (C3), and Jasper (Ja). Most of the isolates cross-reacted with one another to some extent, however, it was felt that the existing differences warranted the separate classifications. Serogroup II contained the single serotype, TV-I. This proposal was amended to suggest that Serogroup I and II be changed to Serogroup A and B. Members of each serogroup would be referred to numerically, i.e. A_1-A_9 for the former, and B_1 for the latter (Hill and Way, 1988; Hill and Way, 1995).

Each serotype is composed of numerous strains or subtypes, defined by their reaction to specified monoclonal antibodies. Under the conventional categorization, VR-299 is placed as a member of the A_1 serotype referred to as West Buxton. The same holds true for the Buhl subtype of the IPN virus.

Buhl Subtype

The IPNV Buhl subtype was first isolated in 1965 from rainbow trout from Buhl, Idaho, and, based on the results of neutralization assays, was placed in the A_1 serotype known as West Buxton, of which VR-299 is the type strain. It is categorized by the American Type Culture Collection as VR-890.

Caswell-Reno *et al.* (1989) used three panels composed of a total of eleven different MAbs to presumptively serotype several strains of IPNV, with each separate panel of MAbs produced against a single virus strain. The Buhl strain of IPNV was found to bind the MAbs to epitopes AS-1, W3, W5, E1, E5, and E6, in contrast to VR-299 which also reacts with epitope W4, and West Buxton (WB) which also reacts with epitopes W1 and W2.

Virulence

Although external conditions such as host and environmental factors play a large part in the course of an IPNV outbreak, another extremely important determinant is the intrinsic virulence of the virus strain involved (Hill, 1982). IPN virus strains display a wide range of virulence levels. For example, in Europe, it has been observed that the Sp serotype of IPNV causes higher mortalities in rainbow trout fry than the Ab serotype (Jorgensen and Kehlet, 1971). Four IPNV strains tested by Sano (1971b) in rainbow trout fry in Japan caused cumulative mortalities ranging from 15-58%. Hill and Dixon (1977) found that three IPNV isolates from the nonsalmonid carriers perch, loach, and carp, caused 6%, 10%, and 60% cumulative mortalities, respectively, when used to challenge susceptible rainbow trout fry. Brook trout fingerlings from Baldwin Mills, Quebec had cumulative mortalities of 16%, 29%, and 31% when infected with difference isolates of IPNV, all belonging to the A_1 serogroup (Silim *et al.*, 1982).

At this time, numerous IPNV isolates have been compiled, representing all of the nine presently recognized serotypes of Serogroup A (P. Reno, personal communication).

These isolates display extremely varied virulence, based on the mortalities caused in susceptible brook trout fry, ranging from 0% (less than or equal to mortalities in control groups) to virtually 100% mortalities (J. Maret, personal communication).

Genomic Sequence

The complete nucleotide sequence of segment A of the IPN virus has been published for the Jasper or Ja (A_9) (Duncan and Dobos, 1986), DRT (A_9) (Chung *et al.*, 1993), Sp (A_2) (Mason, 1992) and N1 (A_2) (Havarstein *et al.*, 1990) strains, representing two different serotypes; while the complete nucleotide sequence of segment B has been published for the Jasper and Sp serotypes (Duncan *et al.*, 1991) only. However, a recent publication (Heppell *et al.*, 1995) discussed the deduced amino acid sequence of the VP2 viral capsid protein of segment A from five additional IPNV strains. The strains analyzed were: VR-299 (A_1), d'Honnincthun or Fr.21 (A_2), Ab (A_3), Hecht or He (A_4), and Canada 2 or C2 (A_7), representing four different serotypes. In addition, nucleotide and amino acid sequence information has been compiled for numerous other IPNV isolates, representing all nine serotypes (B. Nicholson, personal communication).

CHAPTER 3

VIRULENCE COMPARISON OF THREE BUHL SUBTYPE ISOLATES OF INFECTIOUS PANCREATIC NECROSIS VIRUS IN BROOK TROUT (*SALVELINUS FONTINALIS*) FRY

Linda D. Bruslind and Paul W. Reno

Abstract

Infectious pancreatic necrosis virus (IPNV) is an important aquatic pathogen that can cause high mortality in populations of young salmonids. To determine the molecular basis for virulence, brook trout (*Salvelinus fontinalis*) fry were experimentally infected with three different A_1 serotype, Buhl subtype isolates of IPNV. The three isolates were selected based on results of previously completed virulence assays, which indicated that the isolates had significantly differing virulence levels. To confirm this, mortalities from each treatment were recorded for the duration of the experiment (62 days), along with observation of any clinical disease signs. Mortalities began on day 5 post-exposure (dpe), peaked on day 7, then rapidly decreased for all three isolates tested. Diseased fry exhibited whirling, ascites, abdominal hemorrhaging, and prostration on the bottom of the tank. Daily virus titer from live fish was determined for 10 dpe, as well as at 28 and 62 dpe. Viral titers were correlated with fish weight to determine statistical significance. Fish weight was found to negatively correlate to virus titer for the two least virulent isolates. The VP3 region was sequenced for each isolate at two times: before being introduced into the fish (pre-epizootic), and during the epizootic (peak-epizootic). These initial sequencing results demonstrated 100% sequence homology for the viral capsid protein VP3. The VP2 region was sequenced for each isolate at three times: pre-epizootic, peak-epizootic and post-epizootic (2 months after initial exposure to IPNV), in order to determine if major changes existed in the VP2 region that might account for the differences in virulences. Sequence data indicate that two amino acid differences in the VP2 region exist, at residues 217 and 288, distinguishing the least virulent isolates and the most virulent isolate. These amino acid differences might account for the disparity in expressed virulence for these particular IPN virus isolates.

Introduction

Infectious pancreatic necrosis virus (IPNV) causes devastating disease in salmonid fishes worldwide, with the highest levels of mortality occurring in hatchery-reared fish less than 6 months of age. IPNV has been isolated from asymptomatic carrier adult salmonids (Dorson, 1983; Wolf, 1988), as well as from a wide range of non-salmonid fish and shellfish species (Hill, 1982; Hill and Way, 1995).

IPNV is a birnavirus, icosahedral in shape, approximately 60 nm in size, with a genome composed of two segments of dsRNA. Segment A codes for two structural proteins, VP2 and VP3; and a non-structural protease, NS. Segment B codes for the putative RNA-dependent RNA polymerase (Dobos, 1976). An external protein, VP2 stimulates production of type-specific neutralizing monoclonal antibodies or MAbs (Nicholson, 1993), and is believed to contain all the epitopes recognized by neutralizing MAbs (Caswell-Reno *et al.*, 1986; Lipipun *et al.*, 1991; Tarrab *et al.*, 1995).

Under the classification scheme of Hill and Way (1995) there are nine different serotypes of IPNV within the A serogroup. Each serotype is composed of numerous strains which exhibit an extremely wide range of virulence (Hill, 1982; Silim *et al.*, 1982; J. Maret, personal communication). The variation of virulence among isolates may be a reflection of the complex nature of the disease process itself, which is little understood. Although a high mutation rate characterizes the replication of RNA viruses (Holland *et al.*, 1982; Steinhauer and Holland, 1987), it has been found that the serologically related IPN viruses have a high degree of homology, over 96% (Heppell *et al.*, 1993). Recent sequence data from the VP2 viral capsid protein region believed to be responsible for virulence (Sano *et al.*, 1991) also indicates an extreme homology among IPNV isolates (B. Nicholson, personal communication). Therefore, the sequence differences that occur between two closely related isolates, demonstrating markedly differing virulence levels, might pinpoint the amino acid residues that most prominently affect viral performance.

Initial sequencing results demonstrated that the sequence of the viral capsid protein VP3 was identical among isolates, while there were slight differences among isolates in the sequence of the viral capsid protein VP2. Therefore, the sequencing efforts

were focused on the VP2 protein. The purpose of this study was to characterize virulence differences between IPNV isolates, at a molecular level.

Materials and Methods

Experimental Animals

Brook trout (*Salvelinus fontinalis*) were obtained as swim-up fry from the Oregon Department of Fish and Wildlife, Wizard Falls Fish Hatchery, Camp Sherman, OR. IPNV has not been detected at this facility since 1976. The fish were held at the Oregon State University Salmon Disease Laboratory (SDL) facility in Corvallis, OR in UV-irradiated water. Fish were fed Rangen trout starter diet *ad libitum* and averaged 0.08 g in size, initially.

Virus Isolates

Three IPNV Buhl subtype isolates were used in the experiment: CSF 035-85, 91-114, and 91-137. CSF 035-85 was generously donated by Dr. Scott LaPatra of Clear Springs Trout in Idaho. It was isolated in 1985 from rainbow trout fingerlings during an epizootic of IPN disease at Box Canyon, Idaho. The latter two isolates, 91-114 and 91-137, were a kind gift from Kent Hauck at the Idaho Department of Fish and Game. Both of these were isolated in 1991 from asymptomatic carrier adult rainbow trout collected at Sawtooth, Idaho from a blind passage of tissue homogenates. Case # 91-114 was isolated from a single brood Pahsimeroi A strain male, while 91-137 was isolated from four brood Sawtooth A strain males. None of the isolates had been passaged in cell culture more than three times.

The IPNV Buhl subtype isolates were selected after a review of virulence data obtained in previous experiments (J. Maret, personal communication). The isolates were

selected to represent a high virulence isolate (CSF 035-85), a medium virulence isolate (91-114), and a low/no virulence isolate (91-137), based on the mortality previously produced in brook trout fry. In the experiment by J. Maret, the cumulative mortalities caused by the isolates were 99%, 81%, and 33%, respectively. Cumulative mortality observed in control fish was 30%.

The CSF 035-85 isolate was isolated from infected fingerlings (frozen at -70°C for 1 year) with an average titer of $10^{7.25}$ tissue culture infectious dose, 50% endpoint, per milliliter ($TCID_{50}$/ml), and passaged twice in chinook salmon embryo (CHSE-214) cells (Lannan *et al.*, 1984).

The 91-114 and 91-137 isolates were obtained as infected CHSE-214 cell cultures (frozen at -70°C for 1 year) and passaged twice to increase quantity and titrate the viral concentration.

Experimental Exposure of Fish to IPNV

Approximately 400 brook trout fry each were placed into tanks containing 10 L of water at 10°C. Three tanks were used for each treatment (or isolate), and control. Virus was diluted in 20 ml of Hepes-buffered minimum essential medium (HMEM) to give a final tank concentration of 10^5 $TCID_{50}$/ml (McAllister and Owens, 1986). A sample of water from each tank was titered to assure that the correct viral dosage was given. Control fish were immersed in 10 L of water to which 20 ml of HMEM, only, had been added. The fish were held for 5 hours under static flow conditions and supplied with a high volume of air to maintain oxygen levels. After the immersion period, the water in the tanks was replaced with 25 L of virus-free water, the air removed, and the tank water allowed to circulate as usual. All tanks at the SDL were connected to a common flow-through system, with an approximate flow rate of 2.2 L/min. Effluent was discharged through a chlorination system of concentration 2.8 ppm into settling ponds, and ultimately to the Willamette River.

Fish from replicate tanks were combined into a single 100 L tank (flow rate 8.8 L/min) for each isolate and control at 15 dpe, after the primary sample gathering period was completed and it was determined that variability in mortality among replicate tanks was not significantly different.

Sampling Schedule

After exposure to IPNV, five (5) live fish were collected daily from each replicate tank for each isolate during days 1-10 post-exposure. Each sample of five fish was pooled, weighed and homogenized 1:5 in phosphate buffered saline or PBS (0.137 M NaCl, 0.0027 M KCl, 0.0043 M $Na_2HPO_4x7H_2O$, 0.0014 M KH_2PO_4, pH 7.3), for use in determining viral titer. When possible, the fish first selected for viral titering were obviously moribund in appearance.

At 28 dpe, five (5) live fish were collected for each of the three isolates and at 62 dpe, nine (9) live fish were collected for each of the three isolates, for use in determining virus titer. For the 62 dpe sampling, three of the fish sampled were of small size (0.23-0.55 g), three of medium size (1.00-1.48 g), and three of large size (2.02-2.54 g). These fish were titered individually for virus.

Dead fish were collected daily from each tank and recorded over the entire 62 day course of the experiment. Mortalities among replicate tanks (first 15 days post-exposure) and isolates (entire 62 days) were statistically analyzed to determine if there was a significant difference among replicate tanks. The analysis was performed using a generalized linear modeling program (Glimstat, Perth, Australia) after logistic transformation of the mortality data.

Cell Culture and Virus Titration

Virus was propagated in CHSE-214 cells grown in HMEM containing 10% fetal bovine serum (HMEM-10), using 96-well plates, as previously described by Caswell-Reno *et al.* (1986). Samples for virus titer were processed on the same day as collected, within 2 hours, to eliminate loss of virus titer due to freeze/thaw cycles.

The titer of virus in both cell culture and fish tissue was determined using the mathematical model of Spearman-Karber, tissue culture infectious dose, 50% endpoint, per ml ($TCID_{50}$/ml). After determination of the virus titer, any sample remaining was frozen at -70°C and later used for sequencing. Virus titers among replicate tanks and isolates were statistically analyzed by ANOVA to determine if there was a significant difference using the Statview program (Abacus Concepts).

For the first 10 days post-exposure, average viral titers were also compared to corresponding average fish weight (groups of 5 fish each) for replicate tanks of each isolate to determine if there was a statistically significant correlation. For samples collected at 62 dpe, individual viral titer was compared to individual fish weight for each isolate, and statistically analyzed using the linear regression model.

Nucleic Acid Extraction

Viral RNA was extracted from infected cell cultures or directly from fish homogenates using the TRIzol reagent according to manufacturer's instructions (Gibco BRL, Gaithersburg, MD). Details of the nucleic acid procedure are presented in Appendix A. One hundred microliter samples were used with each 1 ml of reagent. After an extraction with chloroform, the RNA was precipitated using sodium acetate and isopropanol. The final pellet was resuspended in a volume of 10 ul RNase-, DNase-free water. The freshly extracted viral RNA was used immediately in a reverse transcriptase assay system.

Primers

Primers to be used for the reverse transcription and polymerase chain reactions were constructed at the OSU Center for Gene Research, based on primers described by Blake *et al.* (1995) for use with the Jasper isolate of IPNV. The 5' primer designed was: 5' TGA GAT CCA TTA TGC TTC C**C**G A 3'. The 3' primer designed was: 5' GAC AGG ATC ATC TTG GCA TAG T 3'. Since the RNA sequence for the Buhl isolate contains an A nucleotide instead of a C at position 20, this nucleotide triphosphate was substituted at this position (nucleotide 20 is indicated above in bold).

Reverse Transcription (RT)

IPNV RNA was extracted from either infected tissue culture cells or fish homogenates as described above and used as a template for the reverse transcriptase reaction. The IPNV RNA was diluted 1:10 in RNase-, DNase-free water, heated at 95°C for 5 min, microfuged briefly, then placed on ice for 2 min.

The following reagents were added together: 1 ul of viral RNA (1:10 dilution), 1 ul of 25mM magnesium chloride ($MgCl_2$), 1 ul (200 units) reverse transcriptase [RT] (Promega, Madison, WI), 1 ul 5x RT buffer (Promega), 1 ul (33 units) RNasin (Promega), 1 ul (55 pmol) 3' primer, 1 ul (55 pmol) 5' primer, 1 ul (1mM each ATP,CTP,GTP,TTP) deoxynucleotides (dNTPs), 12 ul water. The solution was mixed, microfuged briefly, then placed at 37°C for 1 hr to allow reverse transcription to occur. After 1 hr, the mixture was heated at 95°C for 3 min, microfuged briefly, then placed on ice for 2 min. After 2 min, an additional 200 units of RT were added. The solution was mixed, microfuged briefly, and placed at 37°C for an additional hour, to increase the amount of cDNA produced.

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was used to amplify the amount of cDNA produced from the reverse transcription reaction. The 20 ul reaction from the RT was heated at 95°C for 5 min, then placed on ice, to separate the strands.

Then, the following reagents were added: 8 ul 25mM $MgCl_2$, 10 ul 10x PCR buffer (Promega), 1 ul (55 pmol) 3' primer, 1 ul (55 pmol) 5' primer, 0.5 (2.5 units) *Thermus aquaticus* (Taq) polymerase (Promega), 59.5 ul water. The solution was mixed and microfuged briefly. The samples were placed in an Thermolyne Temp-tronic^R (Barnstead/Thermolyne Corporation, Dubuque, IA) for 35 cycles using the following dissociation-annealing program: 94°C - 1 min, 58°C - 1 min, 72°C - 2 min. A 4°C dwell was placed at the end, until samples could be retrieved.

Five microliters of each PCR sample were analyzed in a 1% agarose gel made with TAE buffer (40 mM Tris, 20mM acetic acid, 2 mM EDTA) and ethidium bromide stain. It was electrophoresed at 75 V for 1.5 h, and photographed.

Purification of PCR Products

PCR DNA products were purified using the QIAquick PCR purification kit (Qiagen Inc., Chatsworth, CA) according to manufacturer's instructions. Details of the purification procedure are presented in Appendix A. Briefly, the PCR reaction was diluted 1:10 in buffer, then applied to a QIAquick spin column and microfuged for 1 min. The column was washed with excess buffer and microfuged twice for 1 min each. Each purified PCR preparation was eluted in 30-50 ul of DNase-, RNase-free water. Samples were placed at -70°C for storage until analyzed/sequenced.

Sequencing

Purified PCR products were sequenced by the OSU Center for Gene Research using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) on an automated sequencer. Each virus isolate was sequenced from virus isolated under the following conditions: pre-infection (passaged fewer than three times in CHSE-214 cells), peak of epizootic (directly from day 6-7 infected fish), and post-epizootic (day 62 infected fish). Each sample was sequenced three times, from both the 5' and 3' ends, to ensure sequence accuracy. The sequences were aligned and analyzed using the Genetics Data Environment (GDE) editor, UNIX version (Steven Smith, Harvard Genome Laboratory/ University of Illinois). Nucleotide discrepancies among the three replicates obtained at each sampling point were resolved by determining which sequence represented the majority consensus. After a consensus was determined, the nucleotide sequence was translated into amino acids using the GDE editor.

Protein Secondary Structure

Prediction of protein secondary structure, after conversion of the sequencing results to amino acids, was made using the ProtPlot Program™ (Ross and Golub, 1989).

Results

Mortalities

Acute mortalities characteristic of an IPN epizootic occurred with all three isolates. No elevated mortalities were noted in negative control fish. No signs of disease or deaths were observed in the first four days post-exposure, with the exception of 1 control fish and 1 fish infected with isolate 91-137, both on 2 days post-exposure (dpe).

Daily mortalities for the three isolates and the control fish are charted in Figure 3.1. Moribund fish displaying clinical signs of IPN disease were observed for all three isolates on 5 dpe and mortalities were observed for isolates CSF 035-85 and 91-137. The majority of the moribund fish exhibited typical external and behavioral signs of IPN disease: prostration on the bottom of the tank, rapid gilling, ascites and petechial hemorrhaging in the abdominal area. Whirling of fish was observed infrequently. Mortalities for all three isolates in all nine treatment tanks were observed on 6 dpe. There were no control fish mortalities on this day. Mortalities peaked in the treatment tanks on day 7, then immediately decreased and continued to decline, but did not cease entirely. The experiment was terminated at 62 dpe, with no mortalities occurring in any of the tanks past 56 dpe.

Cumulative mortalities for isolates CSF 035-85, 91-114, and 91-137 at the conclusion of the experiment were 93%, 67% and 78%, respectively. Cumulative mortality for control fish was 3%. Figure 3.2 depicts cumulative mortalities over the course of the experiment. There was a statistically significant difference in cumulative mortalities among isolates. Specifically, there was a significant difference between cumulative mortalities for isolates CSF 035-85 and 91-114, CSF 035-85 and 91-137, but there was not a significant difference in cumulative mortalities between isolates 91-114 and 91-137 (Mantel-Cox $X^2 = 321.5$, 2 d.f.; $p<<0.0001$).

Quantitation of Virus in Exposed Fish

The virus titer of water in each tank at the initiation of the experiment was approximately 10^5 $TCID_{50}$/ml ($10^{4.95}$ - $10^{5.1}$ $TCID_{50}$/ml). The harmonic mean virus titers for each isolate in fish for the first 10 days post-exposure are shown in Figure 3.3. Virus titer increased overall for all three isolates during the first 10 days post-exposure, with a range from 10^5 - 10^{11} $TCID^{50}$/ml. Statistically, there was no significant difference in virus titer among tanks or isolates during the epizootic period (ANOVA $F_{2,89}=0.870$, $p=0.424$).

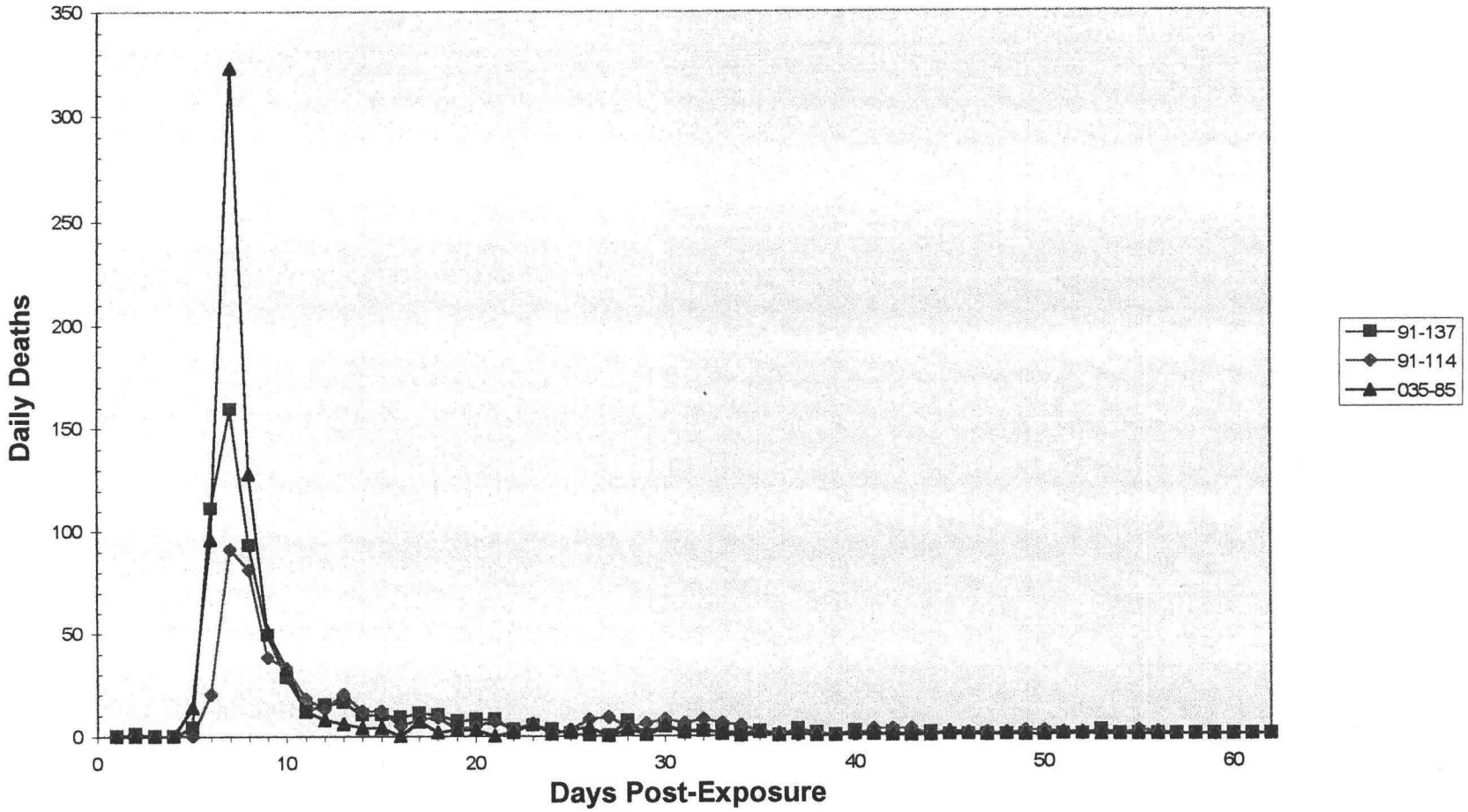

Figure 3.1 Daily mortality of brook trout fry infected with IPNV isolates 91-137, 91-114, and CSF 035-85. Triplicate tanks of approximately 400 hundred fish each were infected with a challenge dose of 10^5 $TCID_{50}$/ml. The average number daily deaths for each virus isolate for all three tanks is shown on the ordinate and plotted against the days post-exposure. The range did not vary significantly among replicate tanks and thus is not shown.

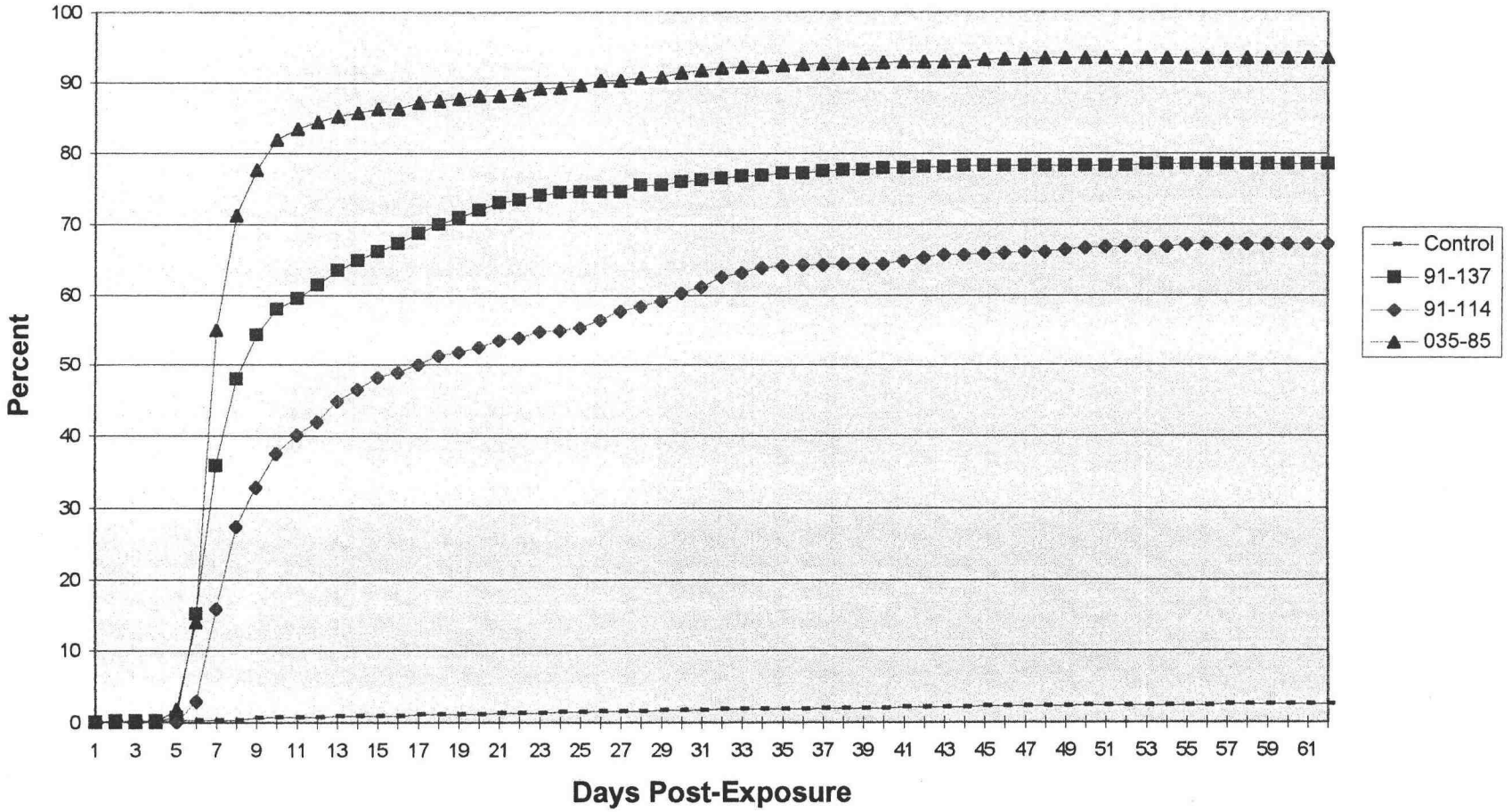

Figure 3.2 Percent cumulative mortality of brook trout fry challenged with IPNV isolates 91-137, 91-114, and CSF 035-85. Triplicate tanks of approximately 400 fish each were infected with a challenge dose of 10^5 $TCID_{50}$/ml. The average percent cumulative mortalities for all three tanks for each virus isolate is shown on the ordinate and plotted against days post-exposure. The range did not vary significantly among tanks and thus is not shown.

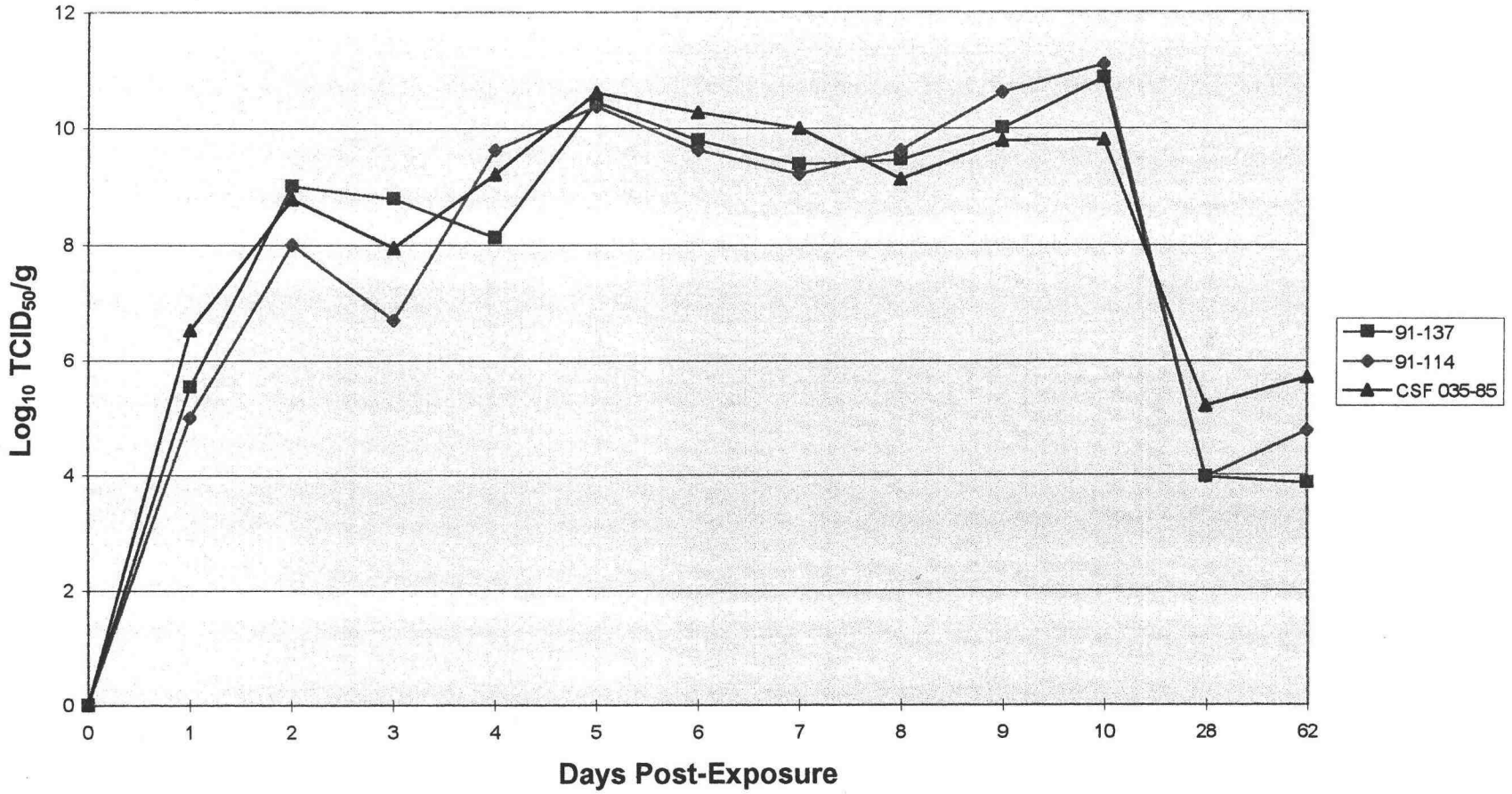

Figure 3.3 Daily virus titer of brook trout fry challenged with IPNV isolates 91-137, 91-114, and CSF 035-85. Triplicate tanks of approximately 400 fish each were infected with a challenge dose of 10^5 $TCID_{50}$/ml. The average daily virus titer as $\log_{10}$ $TCID_{50}$/g for all three tanks for each virus isolate is shown on the ordinate and plotted against days post-exposure. The range did not vary significantly among tanks and thus is not shown.

Virus titers determined at 28 dpe were $10^{3.95}$, $10^{3.95}$, and $10^{5.2}$ $TCID_{50}/g$ for isolates 91-137, 91-114, and CSF 035-85, respectively. This indicated a marked drop in virus titer from the levels seen during the epizootic. Average viral titers at 62 dpe were $10^{3.84}$, $10^{4.76}$, and $10^{5.7}$ $TCID_{50}/g$ for isolates 91-137, 91-114, and CSF 035-85, respectively, which are similar to the 28 dpe virus titer levels. Virus was never detected in control fish.

Weight of Fish vs. Titer

For the first 10 days post-exposure, there was a positive correlation between weight of the fish and virus titer, for all three isolates (Figure 3.4; R^2=0.336, 0.148, 0.070 for isolates 91-137, 91-114, and CSF 035-85, respectively). As the weight of the fish increased, so did the virus titer, per gram of tissue. Size of the fish collected ranged from 0.08-0.23 g over the 10 days, among all three isolates. There was no significant difference between the size of sampled infected fish and the size of control fish.

Similar analysis of samples collected at 62 dpe indicated a significant negative correlation between weight and titer for the isolates 91-114 and 91-137 (Figure 3.5; R^2=0.557 and 0.826, respectively). The smallest fish collected for these isolates carried the highest concentration of virus. Isolate CSF 035-85, the most virulent isolate, did not demonstrate a significant correlation between weight of fish and virus titer (R^2=0.006). Virus titers at 62 dpe for this isolate were statistically similar, regardless of the size of the fish. Size of the fish collected for the isolates ranged from 0.23-2.5 g; with 0.25 g the average size of the smallest fish, 1.5 g the average size of the medium fish, and 2.5 g the average size of the largest fish collected.

Viral RNA and Protein Sequencing

The deduced amino acid sequence of the VP3 protein was constructed at pre- and peak-epizootic, with 100% homology exhibited at both sampling times among all three

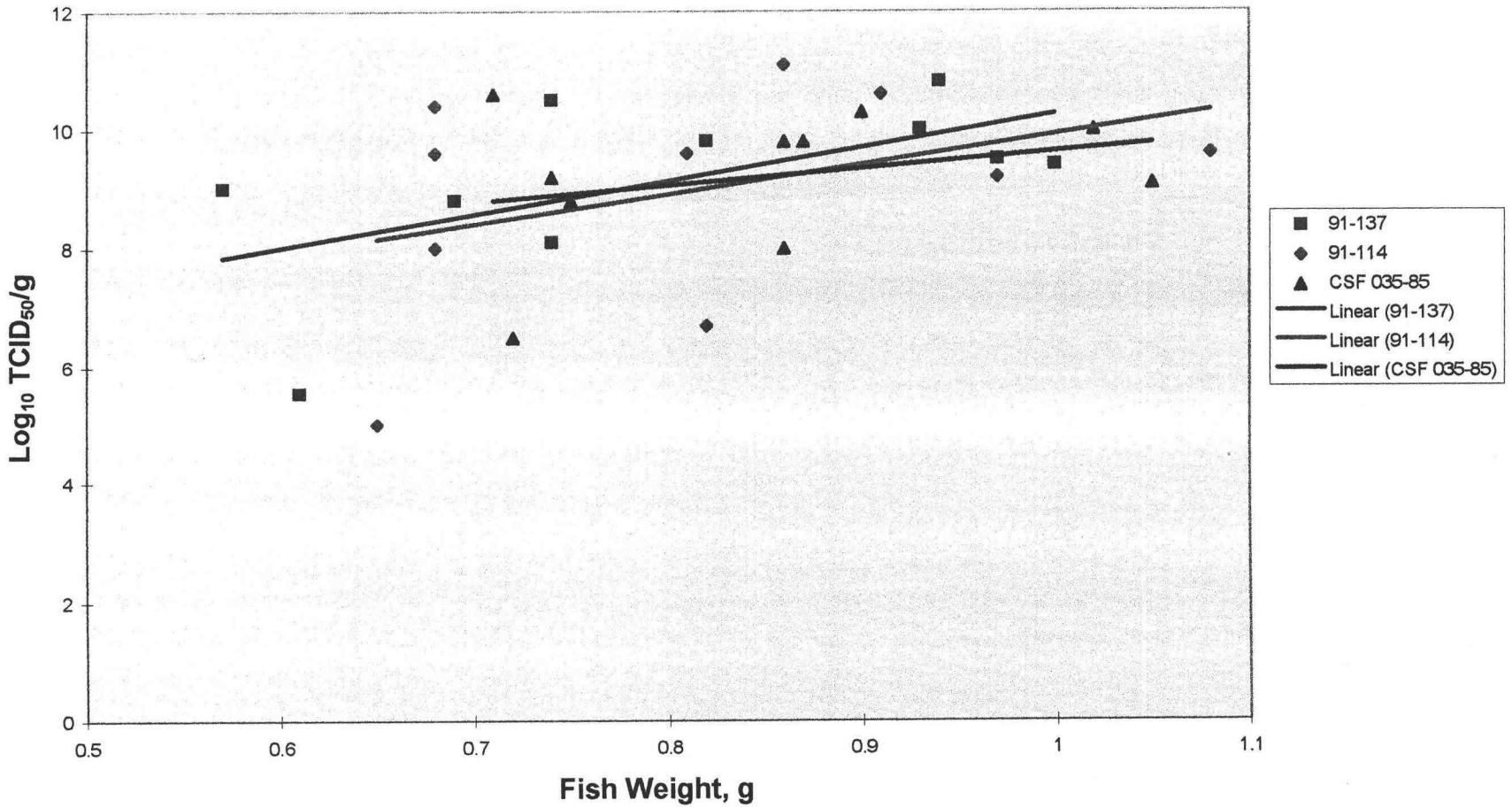

Figure 3.4 Average fish weight (g) versus average virus titer (log_{10} $TCID_{50}$/g) of brook trout infected with IPNV isolates 91-137, 91-114, and CSF 035-85. Fish were sampled at days 1-10 post-exposure. The average virus titer as log_{10} $TCID_{50}$/g for pools of sampled fish is shown on the ordinate and plotted against the average weight of the pooled fish in grams. Linear trendlines are added for each virus isolate.

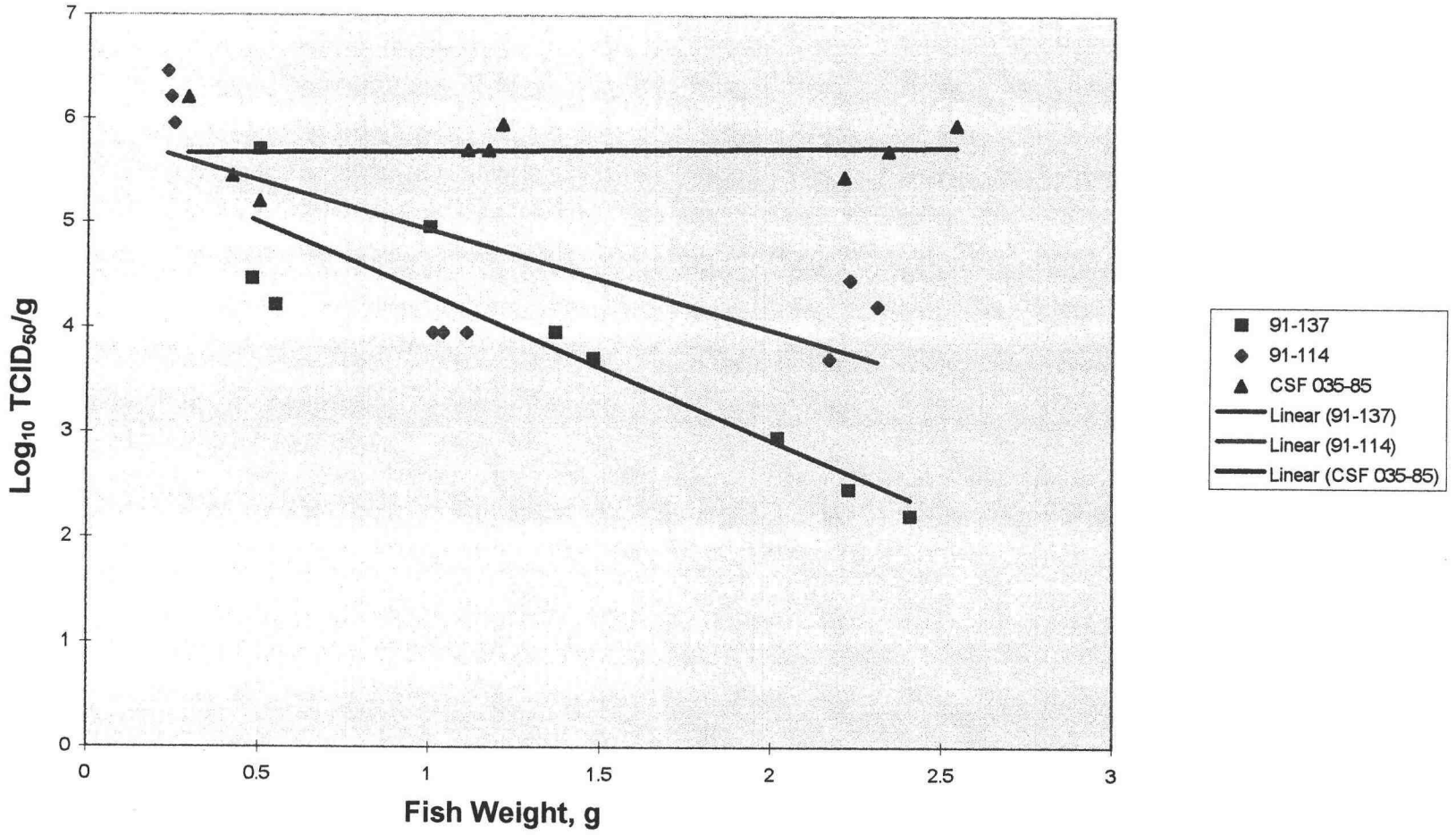

Figure 3.5 Fish weight (g) versus virus titer (log_{10} $TCID_{50}$/g) of brook trout infected with IPNV isolates 91-137, 91-114, and CSF 035-85. Fish were sampled at day 62 post-exposure. The virus titer as log_{10} $TCID_{50}$/g for each sampled fish is shown on the ordinate and plotted against the weight of the fish in grams. Linear trendlines are added for each virus isolate.

isolates (Figure 3.6). The deduced amino acid sequence of the VP2 protein was constructed for the three virus isolates at pre-, peak-, and post-epizootic (Figures 3.7, 3.8, and 3.9). The isolates, which react identically in immunodot blots with a panel of 11 MAbs (J. Maret, personal communication), showed considerable homology of the VP2 amino acid sequence, around 99%. There are two amino acid differences in the VP2 region at positions 217 and 288, between the least virulent isolates (91-114 and 91-137) and the most virulent isolate (CSF 035-85). The differences are demonstrated at all three of the sequence sampling points (pre-, peak, and post-epizootic). At amino acid 217, the 91-114 and 91-137 isolates have an alanine residue, while the CSF 035-85 isolate has a threonine residue. At amino acid 288, the 91-114 and 91-137 isolates have an arginine residue, while the CSF 035-85 isolate has a lysine residue. Although the amino acids belong to different classes, they do not appear to result in a change in the protein secondary structure (Figure 3.10a, b, and c).

There are also two amino acid substitutions at amino acids 194 and 203 that occur for all three isolates, between the pre- and peak-epizootic stages. The arginine residue that was present initially in the pre-epizootic stage changes to a lysine residue by the peak-epizootic stage. This change is still seen when the samples were sequenced for the post-epizootic data and does not appear to affect the secondary structure. There were no sequence changes observed between peak- and post-epizootic samples for any of the isolates.

Additionally, there is one amino acid difference for the 91-114 isolate that is only observed in the pre-epizootic sequencing, at amino acid 256, that does appear to result in a change in the protein secondary structure (Figure 3.10d). The 91-114 isolate has a serine residue amino acid initially, while both of the other isolates have a phenylalanine residue. However, by the time the isolates are sequenced at 7 dpe during the epizootic stage, the serine residue appears to have been replaced by a phenylalanine residue identical to the sequence of the other two isolates.

```
91-137   TASGMDAELQGLLQATMARAKEVKDAEVFKLLKLMSWTRKNDLTD  45
91-114   ********************************************** 45
035-85   ********************************************** 45

91-137   HMYRWSKEDPDAIKFGRLVSTPPKHQEKPKGPDQHTAQEAKATRI  90
91-114   ********************************************** 90
035-85   ********************************************** 90

91-137   SLDAVKAGADFVSPEWIAENNYRGPAPGQFKYYMITGRVPNPGEE 135
91-114   ********************************************** 135
035-85   ********************************************** 135

91-137   YEDYVRKPITRPTDMDKIRRLANSVYGLPHQEPAPDDFYQAVVEV 180
91-114   ********************************************** 180
035-85   ********************************************** 180

91-137   FAENGGRGPDQDQMQDLRDLARQMKRRPRPAETRRQTKTPPRAAT 225
91-114   ********************************************** 225
035-85   ********************************************** 225

91-137   SSGSRFTPSGDDGEV
91-114   ***************
035-85   ***************
```

Figure 3.6 Deduced amino acid sequence of the viral capsid protein, VP3, for the three IPNV isolates 91-137, 91-114, and CSF 035-85. The viral sequences were determined pre-infection, when each virus isolate had been passaged fewer than three times in cell culture. Identical amino acids are indicated by an asterisk (*).

```
91-137  MSTSKATATYLRSIMLPENGPASIPDDITERHILKQETSSYNLEV  45
91-114  ********************************************** 45
035-85  ********************************************** 45

91-137  SESGSGLLVCFPGAPGSRVGAHYRWNLNQTALEFDQWLETSQDLK  90
91-114  ********************************************** 90
035-85  ********************************************** 90

91-137  KAFNYGRLISRKYDIQSSTLPAGLYALNGTLNAATFEGSLSEVES 135
91-114  ********************************************** 135
035-85  ********************************************** 135

91-137  LTYNSLMSLTTNPQDKVNNQLVTKGITVLNLPTGFDKPYVRLEDE 180
91-114  ********************************************** 180
035-85  ********************************************** 180

91-137  TPQGPQSMNGARMRCTAAIAPRRYEIDLPSERLPTVAATATPTTI 225
91-114  **********************************A********* 225
035-85  **********************************T********* 225

91-137  YEGNADIVNSTTVTGDITFQLEAEPANETVFDFILQFLGLDNDVP 270
91-114  ********************************S************* 270
035-85  ********************************F************* 270

91-137  VVTVTSSTLVTVDNHRRASAKFTQSIPTEMITKPITRVKLAYQLN 315
91-114  ****************R*************************** 315
035-85  ****************K*************************** 315

91-137  QQTAIANAATLGANGPASVSFSSGNGNVPGVLPITLVAYEKMTPQ 360
91-114  ********************************************** 360
035-85  ********************************************** 360

91-137  SILTVAGVSNYELIPNPDLLKNMVTKYGKYDPE             393
91-114  ********************************              393
035-85  ********************************              393
```

Figure 3.7 Deduced amino acid sequence of the viral capsid protein, VP2, for the three IPNV isolates 91-137, 91-114, and CSF 035-85. The viral sequences were determined pre-infection, when each virus isolate had been passaged fewer than three times in cell culture. Amino acids that vary among the three isolates are shown, while identical amino acids are indicated by an asterisk (*).

```
91-137   MSTSKATATYLRSIMLPENGPASIPDDITERHILKQETSSYNLEV  45
91-114   *****************************************  45
035-85   *****************************************  45

91-137   SESGSGLLVCFPGAPGSRVGAHYRWNLNQTALEFDQWLETSQDLK  90
91-114   *****************************************  90
035-85   *****************************************  90

91-137   KAFNYGRLISRKYDIQSSTLPAGLYALNGTLNAATFEGSLSEVES  135
91-114   *****************************************  135
035-85   *****************************************  135

91-137   LTYNSLMSLTTNPQDKVNNQLVTKGITVLNLPTGFDKPYVRLEDE  180
91-114   *****************************************  180
035-85   *****************************************  180

91-137   TPQGPQSMNGARMKCTAAIAPRKYEIDLPSERLPTVAATATPTTI  225
91-114   ****************************************A****  225
035-85   ****************************************T****  225

91-137   YEGNADIVNSTTVTGDITFQLEAEPANETVFDFILQFLGLDNDVP  270
91-114   ****************************************F****  270
035-85   ****************************************F****  270

91-137   VVTVTSSTLVTVDNHRRASAKFTQSIPTEMITKPITRVKLAYQLN  315
91-114   ****************R***************************  315
035-85   ****************K***************************  315

91-137   QQTAIANAATLGANGPASVSFSSGNGNVPGVLPITLVAYEKMTPQ  360
91-114   *****************************************  360
035-85   *****************************************  360

91-137   SILTVAGVSNYELIPNPDLLKNMVTKYGKYDPE  393
91-114   *********************************  393
035-85   *********************************  393
```

Figure 3.8 Deduced amino acid sequence of the viral capsid protein, VP2, from the IPNV isolates 91-137, 91-114, and CSF 035-85. The viral sequences were determined at the peak of the epizootic, directly from day 6-7 infected fish. Amino acids that vary among the three isolates are shown, while identical amino acids are indicated by an asterisk (*). Amino acids that changed from the pre-epizootic sampling period are shown in bold.

```
91-137   MSTSKATATYLRSIMLPENGPASIPDDITERHILKQETSSYNLEV  45
91-114   ********************************************  45
035-85   ********************************************  45

91-137   SESGSGLLVCFPGAPGSRVGAHYRWNLNQTALEFDQWLETSQDLK  90
91-114   ********************************************  90
035-85   ********************************************  90

91-137   KAFNYGRLISRKYDIQSSTLPAGLYALNGTLNAATFEGSLSEVES 135
91-114   ********************************************* 135
035-85   ********************************************* 135

91-137   LTYNSLMSLTTNPQDKVNNQLVTKGITVLNLPTGFDKPYVRLEDE 180
91-114   ********************************************* 180
035-85   ********************************************* 180

91-137   TPQGPQSMNGARMKCTAAIAPRKYEIDLPSERLPTVAATATPTTI 225
91-114   ******************************************A**** 225
035-85   ******************************************T**** 225

91-137   YEGNADIVNSTTVTGDITFQLEAEPANETVFDFILQFLGLDNDVP 270
91-114   ********************************************* 270
035-85   ********************************************* 270

91-137   VVTVTSSTLVTVDNHRRASAKFTQSIPTEMITKPITRVKLAYQLN 315
91-114   ****************R************************** 315
035-85   ****************K************************** 315

91-137   QQTAIANAATLGANGPASVSFSSGNGNVPGVLPITLVAYEKMTPQ 360
91-114   ********************************************* 360
035-85   ********************************************* 360

91-137   SILTVAGVSNYELIPNPDLLKNMVTKYGKYDPE             393
91-114   ********************************             393
035-85   ********************************             393
```

Figure 3.9 Deduced amino acid sequence of the viral capsid protein, VP2, from the IPNV isolates 91-137, 91-114, and CSF 035-85. The viral sequences were determined post-epizootic, directly from day 62 infected fish. Amino acids that vary among the three isolates are shown, while identical amino acids are indicated by an asterisk (*). No sequence changes were observed between the peak and post-epizootic sampling periods.

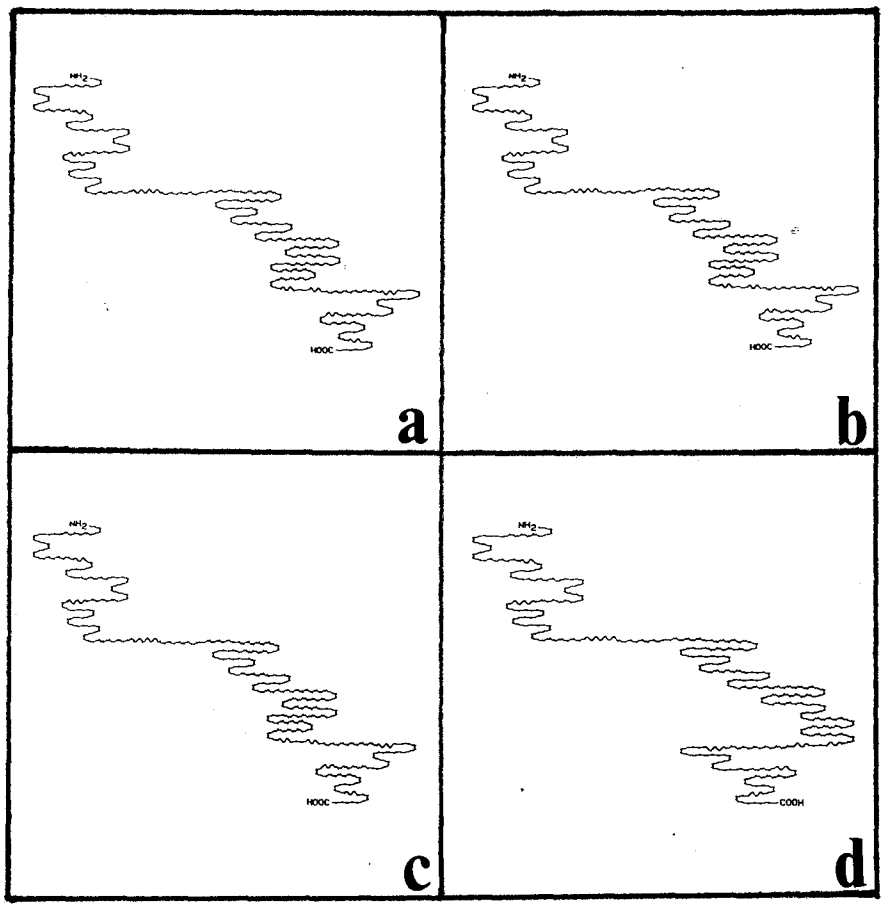

Figure 3.10 Predicted protein secondary structure of the VP2 protein of the IPNV isolates 91-137, 91-114, and CSF 035-85. (a) Virus isolate 91-137, from infected fish at peak and post-epizootic. (b) Virus isolate 91-114, from infected fish at peak and post-epizootic. (c) Virus isolate CSF 035-85, from infected fish at peak and post-epizootic. (d) Virus isolate 91-114, from infected cell culture pre-epizootic.

Discussion

While there were differences in virulence levels among the three isolates, it is unknown at this point why isolate 91-137 caused very low mortality (33%) in brook trout fry in previous experiments (J. Maret, personal communication) and caused 78% mortality in brook trout fry in this experiment. The fry were from the same hatchery, same stock of fish, with a 1 year difference in hatch. There have been a few comparable studies reported in the literature. Working from a hatchery population of brook trout fry, Sonstegard and McDermott (1971) isolated an apparently avirulent IPN virus that induced heavy mortalities when used to experimentally infect another population of brook trout fry. Similarly, Hill (1982) reported isolating an apparently avirulent strain of IPN virus from asymptomatic rainbow trout in England. The IPN viral strain produced heavy mortalities in other rainbow trout fry, as well as in the rainbow trout fry from which it was originally isolated, after experimental challenge. This suggests that it is possible for a virulent strain of IPNV to infect susceptible fry without causing clinical disease. Both of the previously cited cases originated in hatchery fish and the original level of virus exposure is unknown. In any case, there was still a significant difference in the mortalities caused by the isolates 91-137/91-114 versus isolate CSF 035-85, which has consistently caused greater than 90% mortality in experimentally infected brook trout fry less than 6 months of age.

Despite the difference in virulence indicated by the mortality data, there was no significant difference in the amount of virus produced in affected fish for the first 10 days post-exposure. This implies that the virus quantity in fish does not account for differences in virulence between isolates. This concurs with results published by Silim *et al.* (1982), who reported similar virus titers (<1 $\log_{10}$ difference) in several species of trout using virus isolates with differing virulence levels. Therefore, it appears that the ability of the virus to replicate in the host and the efficiency of the host immune response are not related to the ability to kill fish.

It is interesting to note that fish weight was negatively correlated with virus titer for the two less virulent isolates, after the epizootic stage of the infection. It does not

seem likely that the fish are smaller because of the increased amount of virus they are carrying, since the fish infected with the most virulent isolate, CSF 035-85, all had higher virus titers and demonstrated a similar size distribution of fish. It is more likely that these virus isolates have a propensity for more effective replication in smaller fish, possibly because the tissues of these fish are at a stage of more rapid development than the tissues of the larger fish. Alternatively, larger fish may have a better developed immune response and can neutralize virus more effectively. This might explain the fact that IPNV rarely causes epizootics in susceptible fish greater than 6 months of age, with size being a more important factor than age. Okamoto and Sano (1992) concluded that fish body weight was the principle factor influencing mortality of rainbow trout fry experimentally infected with an IPNV Buhl isolate. They observed that the larger fry in groups of infected fish of the same age displayed greater survival rates, over a 2 month sampling period. A similar observation was reported by Biering *et al.* (1994), after experimental infection of small (0.1 g) and large (1.0 g) Atlantic halibut (*Hippoglossus hippoglossus*) fry with an IPNV N1 isolate. At 12°C, the small fry had significantly higher mortality than the other size group and controls. Additionally, the larger fry seemed to clear the IPNV infection after 3 weeks, while the small fry remained IPNV positive during the entire experimental period.

The amino acid difference between virus isolates 91-114/91-137 and CSF 035-85 at residue 217 on protein VP2 is the most likely cause of the significant variation in virulence among the IPNV isolates used for this particular experiment. Although it does not result in a change in the predicted secondary structure of the VP2 protein, the amino acids do belong to different classes. The alanine residue found in 91-114/91-137 is a hydrophobic amino acid, while the threonine residue found in CSF 035-85 is a polar amino acid. Additionally, the site is located in the variable domain of the viral binding region. It has been suggested by Frost *et al.* (1995) that minor changes in the amino acid sequence in this area could result in conformational variation. Pryde *et al.* (1993) found only two amino acid variations after sequencing a Scottish Sp virus strain and comparing the results to the N1 virus strain of Norway, both of which belong to the same serotype. It is possible that the change in amino acids at residue 217 hinders efficient binding of the IPN virus to cell receptors, resulting in a lowered virulence for the less virulent isolates

91-114 and 91-137. The VP2 protein has been implicated previously as an important factor for virus attachment to the cell surface (Darragh and MacDonald, 1982). It is less likely that the amino acid difference at 288 is a factor, since the difference is merely a substitution of one charged amino acid residue for another (arginine versus lysine). However, residues 275 to 288 have been designated a hypervariable region by Heppell *et al.* (1995).

It is equally unlikely that the amino acid changes at 194 and 203 that occur for all isolates between the pre-epizootic sample and the peak/post-epizootic samples are relevant, since the change also involves a substitution of one charged amino acid residue for another (arginine to lysine). The difference between an arginine residue and a lysine residue is one nucleotide, and it is quite possible that a mutation of this sort would occur without affecting the performance of the virus. Since both the changes were from arginine to lysine residues, there is a possibility that the change from replicating in cell culture to fish somehow invoked the change.

The serine residue at position 256 originally sequenced in the 91-114 isolate pre-epizootic sample is interesting, since it is the only change that resulted in a predicted secondary structure alteration. Serine is a polar amino acid, while the phenylalanine found in the other two isolates is a hydrophobic amino acid. Once again, this residue falls within a region designated as a hypervariable region, 243 to 261, by Heppell *et al.* (1995). It is unknown why this alteration would have occurred in the virus in tissue culture, although it appears not be efficacious for the infection of fish, since the residue was changed by 7 dpe. It is possible that this alteration affected binding to particular cells types, since this isolate caused the lowest mortality, particularly in the first 10 days after exposure. By the end of the experiment, 62 days after infection, the mortality caused by isolate 91-114 was not statistically significant from isolate 91-137. This indicates that the alteration found in tissue culture did not permanently affect the isolate.

The data obtained from this experiment point to the possible importance of individual amino acid residues located in the VP2 viral capsid protein hypervariable region, which might account for the widely varying virulence differences displayed among IPNV isolates. The fact that viral replication appears to be influenced partially by

the size of the host, depending on the isolate used, points to another possible connection with viral virulence. This research illustrates the overall complexity of the IPN virus and the ensuing host-viral interactions.

Acknowledgments

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CHAPTER 4

PATHOGENESIS OF INFECTIOUS PANCREATIC NECROSIS DISEASE IN BROOK TROUT (*SALVELINUS FONTINALIS*) FRY DEMONSTRATED USING HISTOPATHOLOGY, IMMUNOHISTOCHEMISTRY, AND *IN SITU* CDNA PROBE HYBRIDIZATION

Linda D. Bruslind and Paul W. Reno

Abstract

Brook trout (*Salvelinus fontinalis*) fry were experimentally infected by immersion with three different Buhl subtype isolates of infectious pancreatic necrosis virus (IPNV), which displayed differing virulence levels. Fish were sampled daily, both before and during the epizootic. Collected samples were fixed, embedded whole, and sectioned. The location in tissues of the IPNV virus was followed for 10 days post-infection (dpe), using histopathology, alkaline phosphatase immunohistochemistry (APIH), and *in situ* hybridization. Histopathology was performed by examining tissue sections stained with hematoxylin and eosin (H&E). APIH was performed using a monoclonal antibody (AS-1) specific to the VP2 viral capsid protein of IPNV, while the *in situ* hybridization assay was performed using cDNA from the VP2 region that had been random labeled with digoxigenin. Microscopic signs of IPN disease were limited primarily to the pancreas and pyloric caeca and were first detected at 5 dpe. Immunohistochemistry and *in situ* hybridization demonstrated virus protein or genomic material in tissues throughout infected fish. The tissue most often containing virus elements, other than the pancreas, appeared to be fibroblasts found in the dermis/hypodermis of the skin, most prominently in the fins. In addition, goblet cells in the gastrointestinal tract contained heavy concentrations of viral antigen. Based on observed results, an IPNV infection appeared to be established in the fish by two routes: the muscle, most likely by entering the lateral line through contact with the water, and from the oral region into the gastrointestinal tract by ingestion.

Introduction

Infectious pancreatic necrosis virus (IPNV) is a significant pathogen of salmonids, particularly of fish less than 6 months of age. IPN viruses cause significant mortalities in

marine and freshwater fish worldwide, with notable losses of both brook and rainbow trout (*Oncorhynchus mykiss*) occurring in eastern and western parts of the U.S.

The characteristic signs of IPN disease include corkscrew whirling about the long axis alternating with prostration, ascites, petechial hemorrhages of ventral areas, castlike excretions from the vent, overall darkening, and rapid respiration (Wolf, 1966). Transmission of IPNV can be vertical or horizontal, and the virus is shed in both feces and sex products of infected fish (Wolf *et al.*. 1963).

Labeled cDNA oligonucleotides have been used previously as diagnostic probes for use in detection of IPNV in infected fish (Christie *et al.*, 1988; Rimstad *et al.*, 1990; Dopazo *et al.*, 1994). However, these methods have not been applied to determine the exact route of viral entry in fish, and much is unknown about the disease process itself. Shankar and Yamamoto (1994) determined the specific location of viral replication of a strain of IPNV from lake trout (*Salvelinus namaycush*) by assaying the fish organs individually. While this method indicates what tissue is positive for virus, it does not conclusively demonstrate the course of the virus in the positive tissues. Swanson *et al.* (1982) used virus isolation (based on titer), histopathology, and immunofluorescence simultaneously to follow the distribution of the IPN virus in young brook trout fingerlings that had been artificially infected by intraperitoneal inoculation. This experiment demonstrated the process of viral infection in fish tissue, but did not imitate the possible occurrences of a natural infection.

The use of molecular and immunohistochemical probes allowed elucidation of the specific route by which the IPNV virus enters the host, with correlation to the tissue damage involved by using classical histopathological techniques.

Materials and Methods

Experimental Animals

Brook trout (*Salvelinus fontinalis*) were obtained as swim-up fry from the Oregon Department of Fish and Wildlife, Wizard Falls Fish Hatchery, Camp Sherman, OR. No IPNV has been detected at this facility since 1975 (C. Banner, personal communication). The fish were held at the Oregon State University Salmon Disease Laboratory (SDL) facility in Corvallis, OR. Fish were fed Rangen trout starter diet *ad libitum* and averaged 0.08 g in size, initially.

Virus Isolates

Three IPNV Buhl subtype isolates were used in the experiment: CSF 035-85, 91-114, and 91-137. CSF 035-85 was generously donated by Dr. Scott LaPatra of Clear Springs Trout Co., Buhl, Idaho. It was isolated in 1985 from rainbow trout fingerlings during an epizootic of IPN disease in Idaho. The latter two isolates, 91-114 and 91-137, were a kind gift from Kent Hauck at the Idaho Department of Fish and Game. Both were isolated in 1991 from asymptomatic carrier adult rainbow trout collected at Sawtooth, Idaho from a blind passage of tissue homogenates. Case number 91-114 was isolated from a single brood Pahsimeroi A strain male, while 91-137 was isolated from four brood Sawtooth A strain males. None of the isolates had been passaged in cell culture more than three times.

The IPNV Buhl subtype isolates were selected after review of virulence data obtained in previous experiments (J. Maret, personal communication). The isolates were selected to represent a high virulence isolate (CSF 035-85), a medium virulence isolate (91-114), and a low/no virulence isolate (91-137), based on the mortality previously produced in brook trout fry. In the experiment by J. Maret, the cumulative mortalities

caused by the isolates were 99%, 81%, and 33%, respectively. Cumulative mortality observed in control fish was 30%.

The CSF 035-85 isolate was isolated from infected fingerlings (frozen at -70°C for 1 year) with an average titer of $10^{7.25}$ tissue culture infectious dose, 50% endpoint, per milliliter ($TCID_{50}$/ml), and passaged twice in chinook salmon embryo (CHSE-214) cells (Lannan *et al.*, 1984).

The 91-114 and 91-137 isolates were obtained as infected CHSE-214 cell cultures (frozen at -70°C for 1 year) and passaged twice to increase quantity and titrate the viral concentration.

Experimental Exposure of Fish to IPNV

Approximately 400 brook trout fry each were placed into tanks containing 10 L of water at 10°C. Three tanks were used for each treatment (or isolate), and control. Virus was diluted in 20 ml of Hepes-buffered minimum essential medium (HMEM) to give a final tank concentration of 10^5 $TCID_{50}$/ml (McAllister and Owens, 1986). A sample of water from each tank was titered to assure the correct viral dosage was given. Control fish were immersed in 10 L of water to which 20 ml of HMEM, only, had been added. The fish were held for 5 hours under static flow conditions and supplied with a high volume of air to maintain oxygen levels. After the immersion period, the water in the tanks was replaced with 25 L of virus-free water, the air removed, and the tank water allowed to circulate as usual. All tanks at the SDL were connected to a common flow-through system, with an approximate flow rate of 2.2 L/min. Effluent was discharged through a chlorination system of concentration 2.8 ppm into settling ponds, and ultimately to the Willamette River.

Fish from replicate tanks were combined into a single 100 L tank (flowrate 8.8 L/min) for each isolate and control at 15 dpe, after the primary sample gathering period was completed and it was determined that variability in mortality among replicate tanks was not significantly different.

Sampling Schedule and Preparation

Ten (10) live fish were collected daily from each tank for the first 10 days following exposure to IPNV. When possible, the fish first selected for sampling were exhibiting slight clinical signs of IPN disease. Two (2) fish exposed to each isolate were collected at 62 dpe, immediately preceding termination of the experiment. Fish were preserved whole in 10% buffered formalin, embedded in ParaPlast Plus paraffin (Fisher Scientific, Pittsburgh, PA) and sectioned longitudinally at 5-7 um thickness on a rotary microtome. The sections were placed on positively charged slides (Fisher Scientific) and air dried. Tissue sections were rehydrated by passing the slides through a series of graded alcohols (absolute to 50%), and finally into distilled water.

Histopathology

Rehydrated tissue sections were stained with Mayer's hematoxylin, then counterstained with eosin. Sections were dehydrated in a series of graded alcohols (50% to absolute), then cleared with Slide Brite. Slides were mounted with a coverslip using Permount mounting medium. Sections were examined using normal bright field microscopy to determine the extent and location of any tissue damage.

The degree of IPNV infection in individual tissues was rated on an intensity scale of 1 to 4. For histopathology, intensity was measured by the level of damage sustained to the tissue. Intact tissues that showed barely discernible signs of infection were rated a 1, while moderate damage was rated a 2. Heavy tissue damage was rated a 3, and complete tissue destruction was rated a 4.

Probe Development

Viral cDNA of the VP2 viral capsid protein region of all three isolates was transcribed and amplified using the reverse transciptase polymerase chain reaction (RT-PCR) on infected fish homogenates. The cDNA was purified using the QIAquick purification for PCR products (Qiagen, Chatsworth, CA), quantified on a 1% agarose gel made with TAE buffer (40 mM Tris, 20mM acetic acid, 2 mM EDTA) and ethidium bromide stain, then used as a template for the labeling reaction. The cDNA was random prime labeled with digoxigenin, using a labeling kit according to manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). Purified template was boiled, then placed on ice. The hexanucleotide and nucleotide labeling mixtures were added to the template, along with Klenow enzyme, and incubated at 37°C overnight. After incubation, the labeled DNA was precipitated with lithium chloride and ethanol, and the pellet resuspended in 50 ul of tris-EDTA (TE) buffer. Labeled probes were diluted and tested using a direct detection procedure to determine the concentration of the labeling.

In Situ Hybridization

Tissue sections were probed with digoxigenin-labeled IPNV cDNA and a detection kit (Boehringer Mannheim), using the method described by Bruce *et al.* (1993). Sections were rehydrated, then washed in water and phosphate buffered saline (PBS: 0.137 M NaCl, 0.0027 M KCl, 0.0043 M $Na_2HPO_4x7H_2O$, 0.0014 M KH_2PO_4, pH 7.3), followed by a proteinase K treatment. The proteinase K was inactivated with PBS containing 0.2% glycine. The sections were prehybridized for 1.5 h at room temperature. A Thermolyne Amplitron^R II (Barnstead/Thermolyne Corporation, Dubuque, IA) with an *in situ* plate accessory was used to heat the slides to 95°C for 3 min immediately following addition of the probe, before hybridization overnight at 37°C. After hybridization, sections were washed in decreasing concentrations of sodium citrate (SSC) buffer, then blocked with normal sheep serum (NSS) and Triton X-100. The alkaline-

phosphatase-digoxigenin conjugate (Boehringer Mannheim) was diluted 1:5000 with NSS and Triton X-100, then placed on sections for 45 min at room temperature. Slides were washed, and the color reagent (Boehringer Mannheim) placed on overnight. After the *in situ* hybridization procedure was complete, sections were rinsed in water and counterstained using nuclear fast red (Vector Laboratories, Burlingame, CA), and mounted with a coverslip. Tissue sections were examined using normal bright field light microscopy for cells or tissue displaying a dark blue to purple precipitate indicating the presence of homologous IPNV RNA.

For the *in situ* hybridization assay, intensity was measured based upon the strength of the positive reaction observed, compared to background observed in control tissue. A light purple, barely discernible reaction was rated a 1, while a more definite reaction was rated a 2. Tissue that had a dark, intense purple reaction were rated a 3-4, depending on the proportion of tissue affected.

Alkaline Phosphatase Immunohistochemistry (APIH)

IPNV antigen was detected in paraffin embedded fish tissues using the Vectastain^{R} alkaline phosphatase substrate kit (Vector Laboratories) and the IPNV VP2-specific monoclonal antibody AS-1 (Caswell-Reno *et al.*, 1989) made in mice, with a method similar to that described by Drolet *et al.* (1994). Rehydrated sections were washed in water, then dipped in 20% acetic acid at 4°C for 15 seconds to eliminate any alkaline phosphatase activity associated with the tissue. Sections were rinsed in water, followed by PBS, then blocked with 2% normal serum followed by 5% nonfat milk. Sections were incubated for 1 h with undilute AS-1 antibody prepared in cell culture medium, rinsed, then incubated with biotinylated anti-mouse secondary antibody (Vector Laboratories). Sections were rinsed again before the avidin-biotin complex (Vector Laboratories) was added. Sections were rinsed, equilibrated, then incubated with the Vectastain^{R} blue substrate components (Vector Laboratories) for 40 min at 37°C. The

tissue was counterstained using nuclear fast red (Vector Laboratories) and mounted under a coverslip. Tissue sections were examined using normal bright field light microscopy for cells or tissue displaying a bright blue precipitate indicating the presence of IPNV antigen.

For the APIH assay, intensity was also measured based upon the strength of the positive reaction observed, compared to background observed in control tissue. Tissues that stained a light blue were given a rating of 1-2, while tissues staining a bright blue were given a rating of 3-4, once again depending upon the proportion of tissue affected.

Results

Virus Infection

Mortalities and signs of IPN disease were first observed on day 5 post-exposure (dpe), proceeded to peak on 7 dpe, then decreased with time (Figure 4.1). Cumulative percent mortality at 62 dpe was 93%, 78% and 67% for isolates CSF 035-85, 91-137 and 91-114, respectively. Cumulative mortality for control fish was 3%.

Moribund fish, first observed on 5 dpe as well, demonstrated classical clinical signs of IPNV and were observed to be prostrate on the bottom of the tank, dark in color, and respiring rapidly; many had ascites and petechial hemorrhaging in the abdominal area. Whirling of fish was observed infrequently. Control fish were not observed to demonstrate disease signs at any time during the experiment.

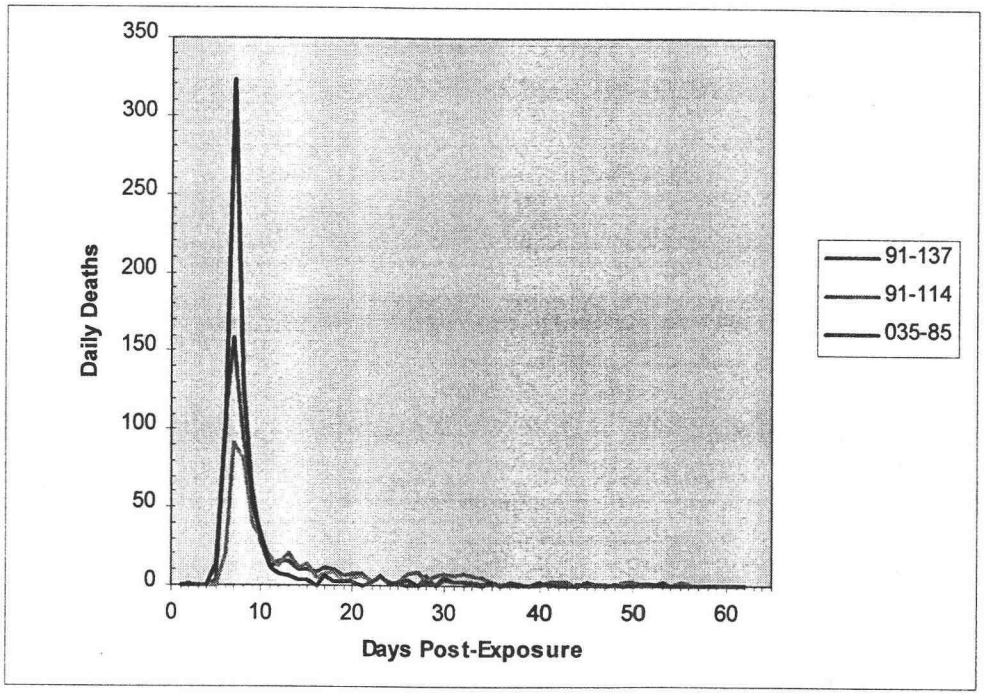

Figure 4.1 Mortality curve of brook trout (*S. fontinalis*) fry after artificial infection by immersion with IPNV Buhl subtype isolates 91-137, 91-114, and CSF 035-85. The pre-epizootic period is from day 0-5 and the epizootic period is from day 6-10.

Histopathology

Observation of fish tissue sections stained with H&E indicate that lesions associated with IPN disease were evident in the tissue of infected fish before the time that the peak infection occurred; that is, before the time when the largest number of fish begin to die from IPN disease (7 dpe). No signs were observed in the tissue of sampled control fish during the course of the experiment (Figure 4.2a)

A few possible signs of IPN infection were first observed in fish tissue on 3 dpe for all three isolates, before fish began exhibiting clinical signs of infection. Signs were limited to the pancreatic acinar cells and consisted of small areas of focal necrosis with pyknosis and karyorhexis (Figure 4.2b).

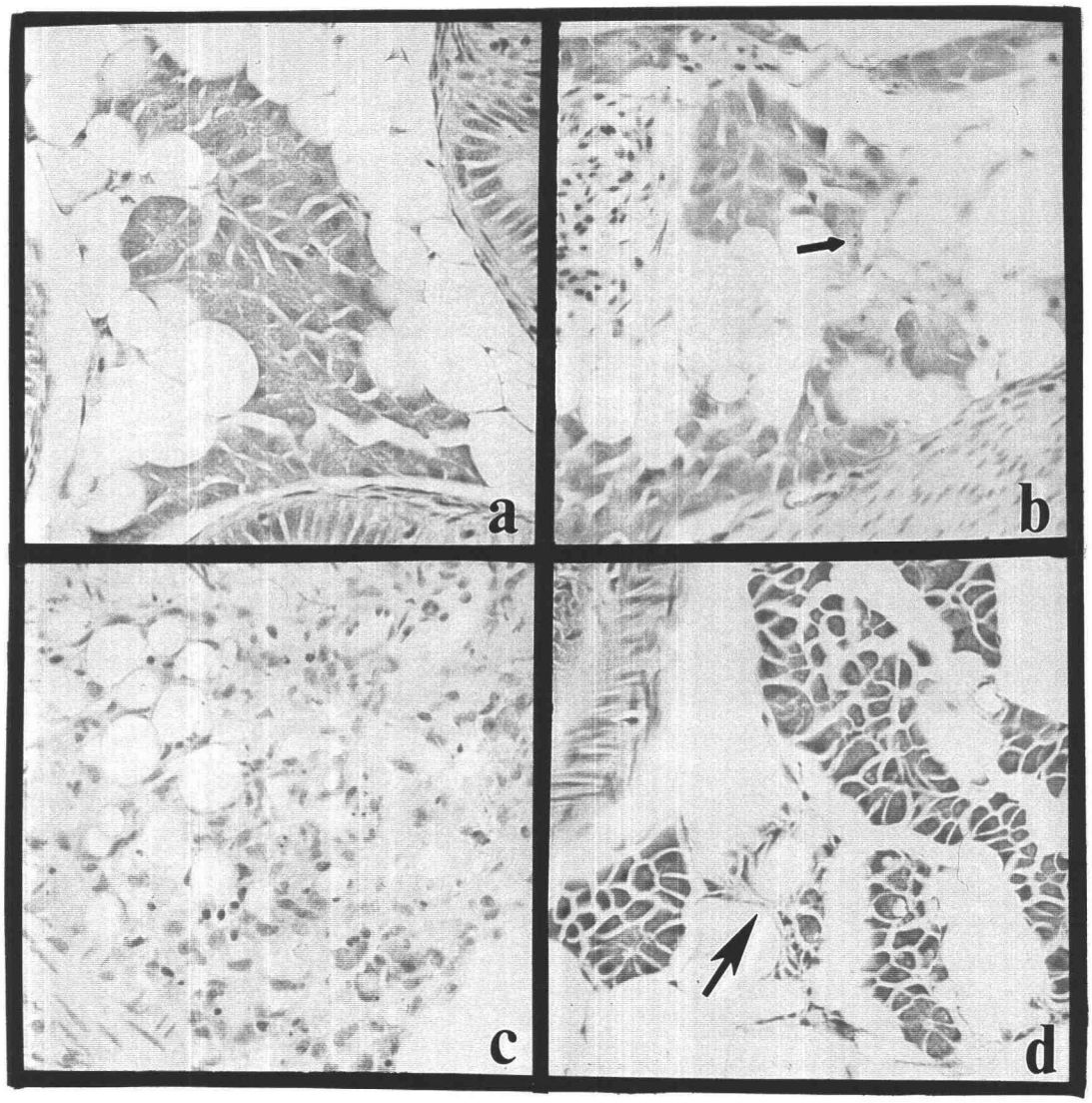

Figure 4.2 Histopathology of tissue sections from brook trout fry infected with IPNV. Sections were stained with Mayer's hematoxylin and eosin. (a) Pancreatic tissue of control tissue (250x). (b) Pancreatic tissue at 4 dpe, showing slight pathological signs of IPNV, such as pyknotic nuclei (arrow) (250x). (c) Pancreatic tissue at 9 dpe, showing extreme signs of necrosis and tissue disruption (250x). (d) Pancreatic tissue at 62 dpe showing a small area of necrosis (arrow) surrounded by normal acinar cells (250x).

On 4 dpe, the IPN disease signs observed in the tissue from fish infected with isolate 91-114 still consisted of limited areas of cellular necrosis in the pancreatic acinar cells. The tissue of fish infected with virus isolates 91-137 and CSF 035-85 were showing moderate signs of IPN infection. Areas with obvious cellular disruption and tissue disorganization were evident in the pancreatic acinar cells, as well as areas of slight necrosis. Pyknotic nuclei and karyorhexis were evident, and some sections had infiltration by lymphocytes.

By 5-7 dpe, IPN lesions were obviously visible in pancreatic tissue from fish infected with each of the three isolates. They consisted primarily of cellular disruption, degeneration of tissue structure, nuclear pyknosis, varying degrees of hemorrhage, and areas of acute cytolytic necrosis. Intact acinar cells were dark and rounded, indicating some level of degeneration. Some sloughing of the pyloric caeca and intestinal mucosal epithelium was visible.

Signs of IPN lesions reached their peak in all tissue from fish infected with each of the three isolates at 8-10 dpe (Figure 4.2c). Massive cellular disruption was evident, along with hemorrhaging and necrosis. Many fish had little or no unaffected pancreatic tissue remaining at this time. Severe sloughing of the pyloric caeca and mucosal epithelium of the intestine were evident. Most of the IPN signs were confined to the pancreas, pyloric caeca, and intestine, with the exception of small areas of hyaline muscle degeneration observed in several of the sampled fish.

Histopathology of fish sampled at 62 dpe showed large areas of necrosis throughout the pancreas (not shown), however, many areas of unaltered pancreas with intact acinar cells remained (Figure 4.2d). Fish sampled at this time did not show any clinical signs of IPN disease and outwardly appeared to be healthy.

There was no obvious difference noted histologically in the degree of tissue damage caused by the various IPNV isolates.

In Situ Hybridization of IPNV RNA

In order to determine the location of IPNV genomic RNA in tissues of infected brook trout, a cDNA probe was used. A blue-purple precipitate indicating the presence of viral RNA was observed in the infected fish tissue as early as 1 dpe, the first sampling date, for all three isolates. For fish exposed by immersion to virus isolate 91-114, the IPNV RNA was initially confined to the skin epidermis, hyaline cartilage of the nose, and ocular lens of infected fish tissue. For fish exposed by immersion to virus isolates 91-137 and CSF 035-85, viral nucleic acid was much more widespread, occurring in the mucosal epithelium of the esophagus, stomach and pyloric caeca, as well as the striated musculature of the myomeres (Figure 4.3a). Fish exposed to virus isolate CSF 035-85 also had IPNV RNA in the skin epidermis and ocular lens, in addition to the mucosa of the intestine. Reactions, based on intensity and overall percent of tissue infected, were generally light to moderate at 1 dpe.

As the infection progressed, the intensity and location of positive reactions generally increased for all three isolates. Viral nucleic acid was occasionally present in the 10 days post-exposure in the following tissues: intestine, kidney, spleen, brain, liver, eye, cartilage, swim bladder, and thymus. Reactions occurring in these tissues were sporadic, usually affecting single fish of 10 replicates, and highly variable in intensity, regardless of the virus isolate used to infect the fish orignally. A positive reaction in the thymus was only observed in a single fish, infected with virus isolate CSF 035-85 (6 dpe).

The most consistently affected tissues with the largest amount of viral RNA were: the pancreas and adjoining adipose cells, stomach, pyloric caeca, gills, heart, muscle, and epithelial skin and dermis/hypodermis.

Using the mean of the intensity scores given to the hybridization reaction in these tissues for the ten fish examined daily for each virus isolate, the progression of the infection could be charted (Figures 4.4, 4.5, 4.6). Just prior to and during the epizootic

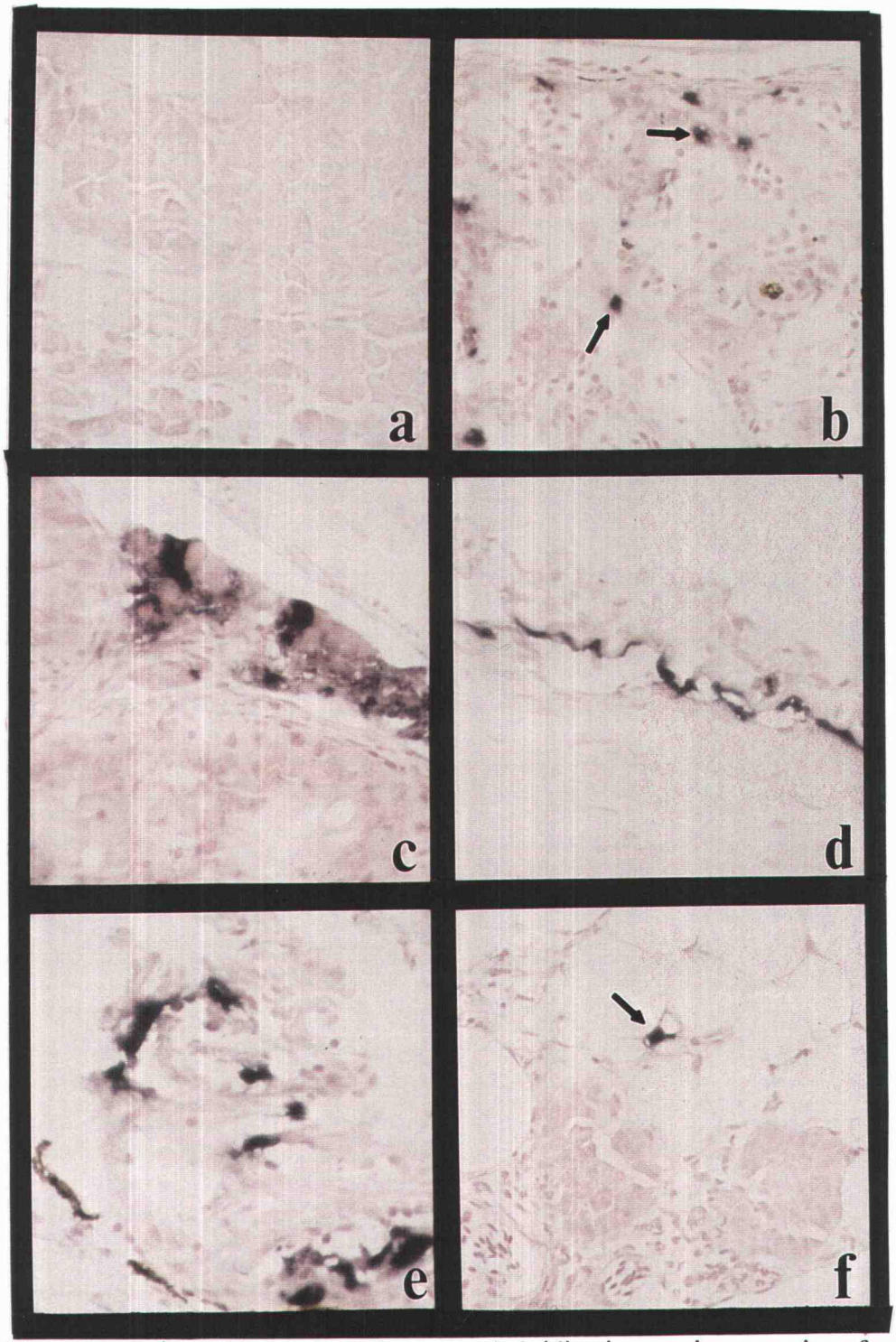

Figure 4.3 Detection of IPNV RNA by *in situ* hybridization on tissue sections from infected brook trout fry (nuclear fast red counterstain). (a) Pancreatic tissue of control fish (250x). (b) Limited positive reaction in the kidney (arrow) (250x). (c) Intense positive reaction in pancreatic tissue (250x). (d) Intense positive reaction in the epithelial dermis/hypodermis (250x). (e) Intense positive foci in heart cells (250x). (f) Individual pancreatic cell showing a positive reaction from a fish sampled at 62 dpe (250x).

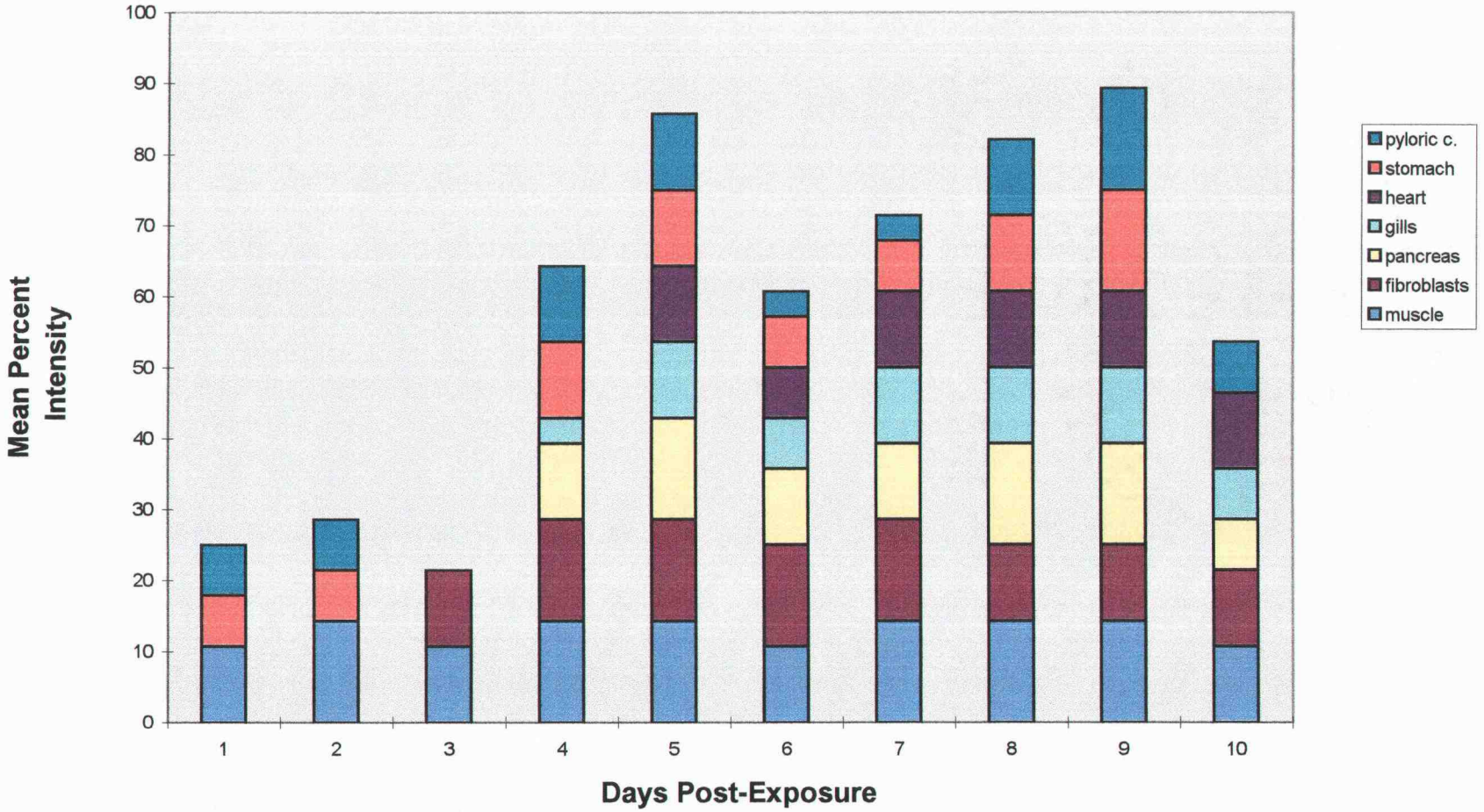

Figure 4.4 Intensity of *in situ* hybridization reaction in tissue sections from fish infected with IPNV isolate 91-137. Each individual tissue type was given an intensity rating from 1-4, based on the positive reaction observed. The total mean percent intensity, based on the number of tissues examined, is shown on the ordinate and plotted against days post-exposure.

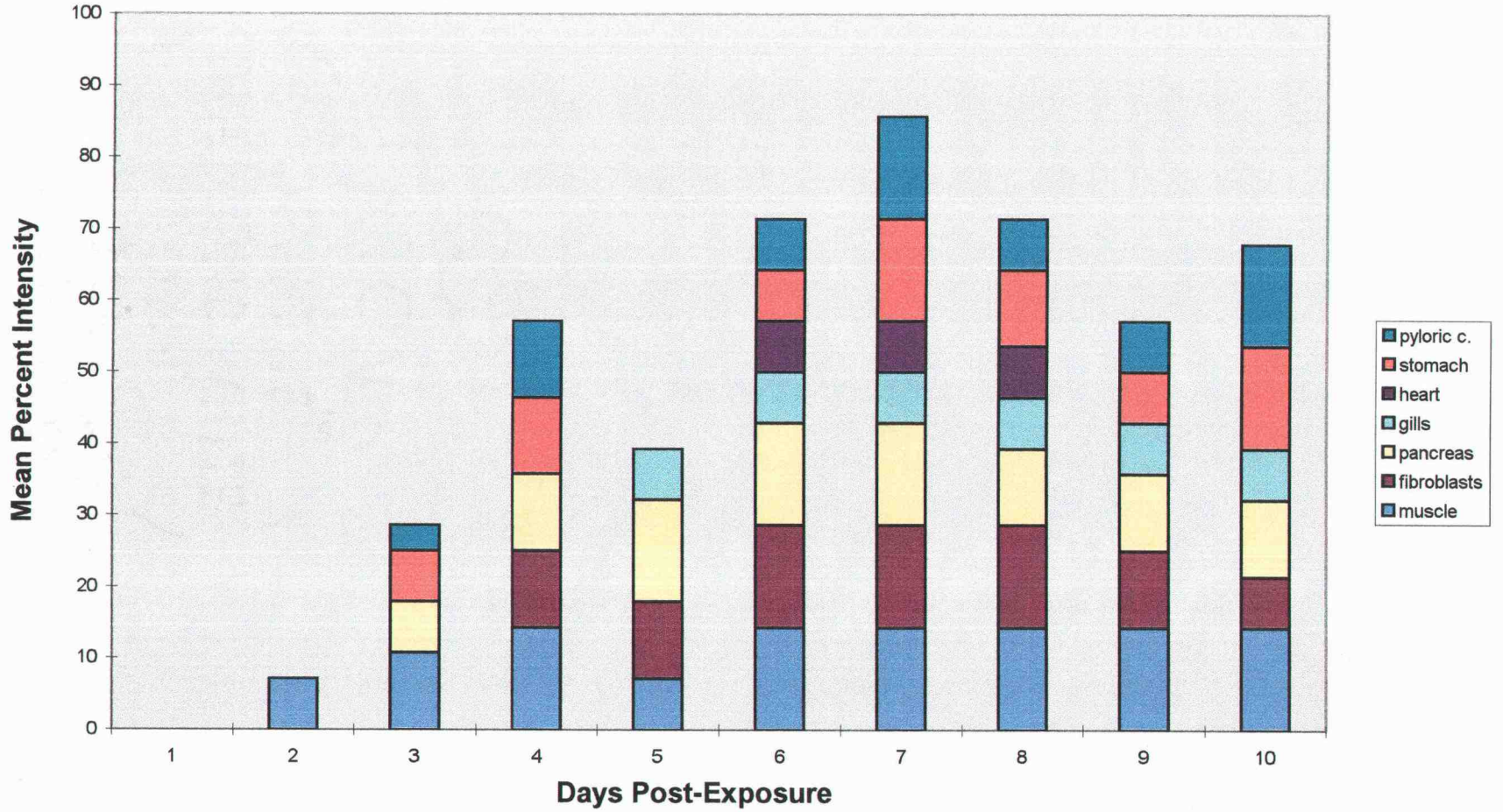

Figure 4.5 Intensity of *in situ* hybridization reaction in tissue sections from fish infected with IPNV isolate 91-114. Each individual tissue type was given an intensity rating from 1-4, based on the positive reaction observed. The total mean percent intensity, based on the number of tissues examined, is shown on the ordinate and plotted against days post-exposure.

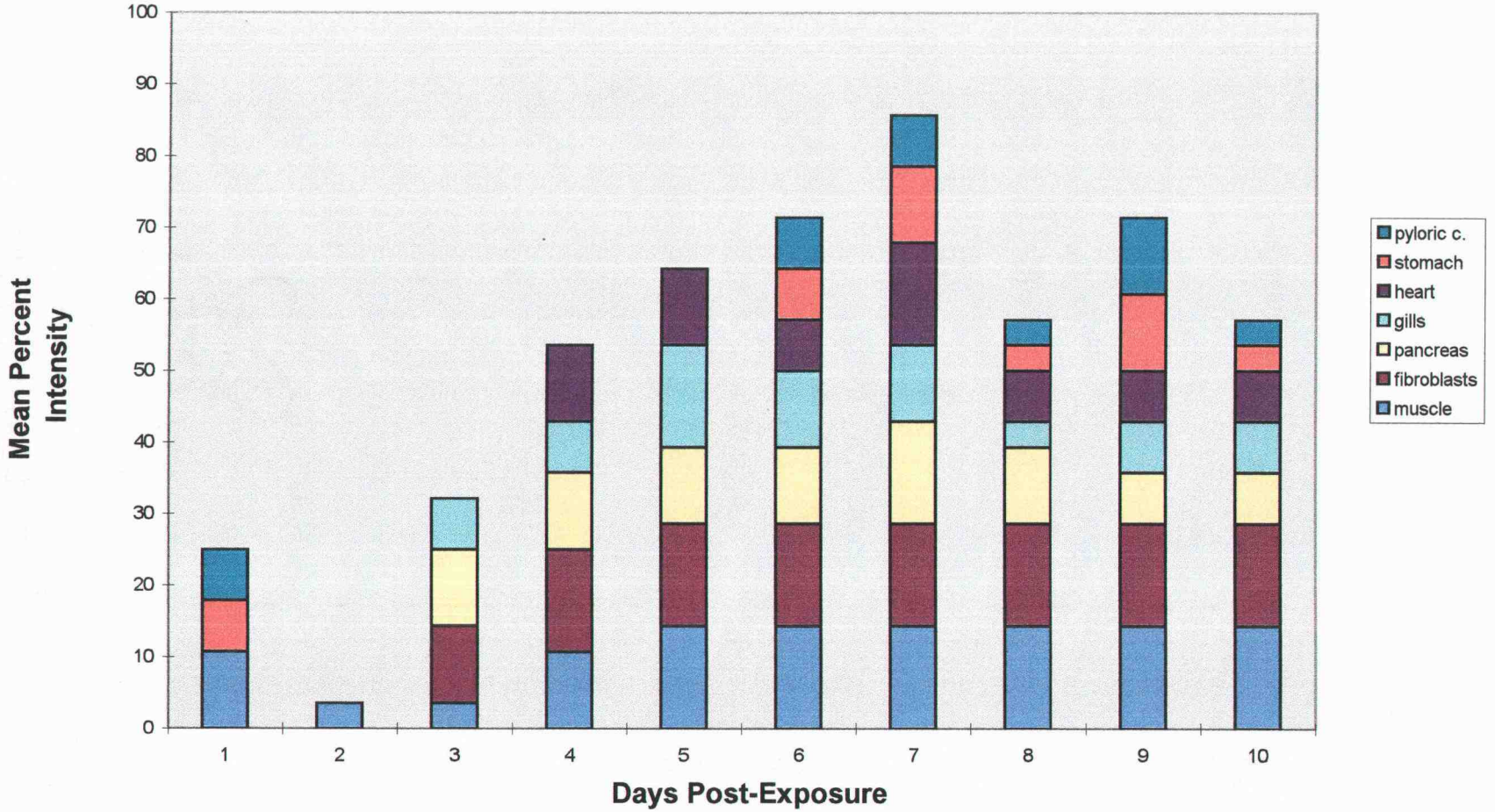

Figure 4.6 Intensity of *in situ* hybridization reaction in tissue sections from fish infected with IPNV isolate CSF 035-85. Each individual tissue type was given an intensity rating from 1-4, based on the positive reaction observed. The total mean percent intensity, based on the number of tissues examined, is shown on the ordinate and plotted against days post-exposure.

stage of the infection, a particularly strong reaction was observed in what appeared to be fibroblasts located in the dermis/hypodermis of the fins and epithelial skin (Figure 4.3d), for fish exposed to any of the three virus isolates. Also during the epizootic stage of the infection, sloughed cells in the lumen of the intestine displayed a very strong positive reaction, indicating the presence of virus and/or viral RNA. However, the presence of a large amount of viral RNA in various tissues did not correlate with observed lesions by histopathology.

In the initial stages of the infection, positive reactions for fish exposed to any of the three virus isolates were diffuse in nature, appearing as a light to moderate purple color located generally throughout the affected tissue. As the infection progressed to the epizootic stage, positive reactions became much more intense and focal in appearance, affecting individual clusters of cells (Figure 4.3e). This change in the reaction was observable on a day to day basis, for each isolate individually. The change from diffuse positive to focal reactions first occurred for fish exposed to virus isolate CSF 035-85 at 3 dpe, for fish exposed to virus isolate 91-137 at 4 dpe, and for fish exposed to virus isolate 91-114 at 6 dpe.

Fish sampled at 62 dpe had only a small amoung of viral RNA in limited areas of the tissue of the pancreas, pyloric caeca, kidney, skin, and muscle. Reactions consisted of only a few foci per infected tissue (Figure 4.3f), which was scored a 1-2 based on intensity and extent of tissue affected.

Alkaline Phosphatase Immunohistochemistry (APIH)

Staining reactions observed using the APIH procedure correlated well with the results obtained with the *in situ* hybridization procedure. The same positive tissues were observed, with slight variations in intensity, with one exception. A positive reaction using APIH was observed during the epizootic period (5-10 dpe) in goblet mucous cells of the gastrointestinal tract and epithelium of the skin, that was only marginally visible using *in situ* hybridization. The reaction was quite strong (Figure 4.7a), and was not observed in

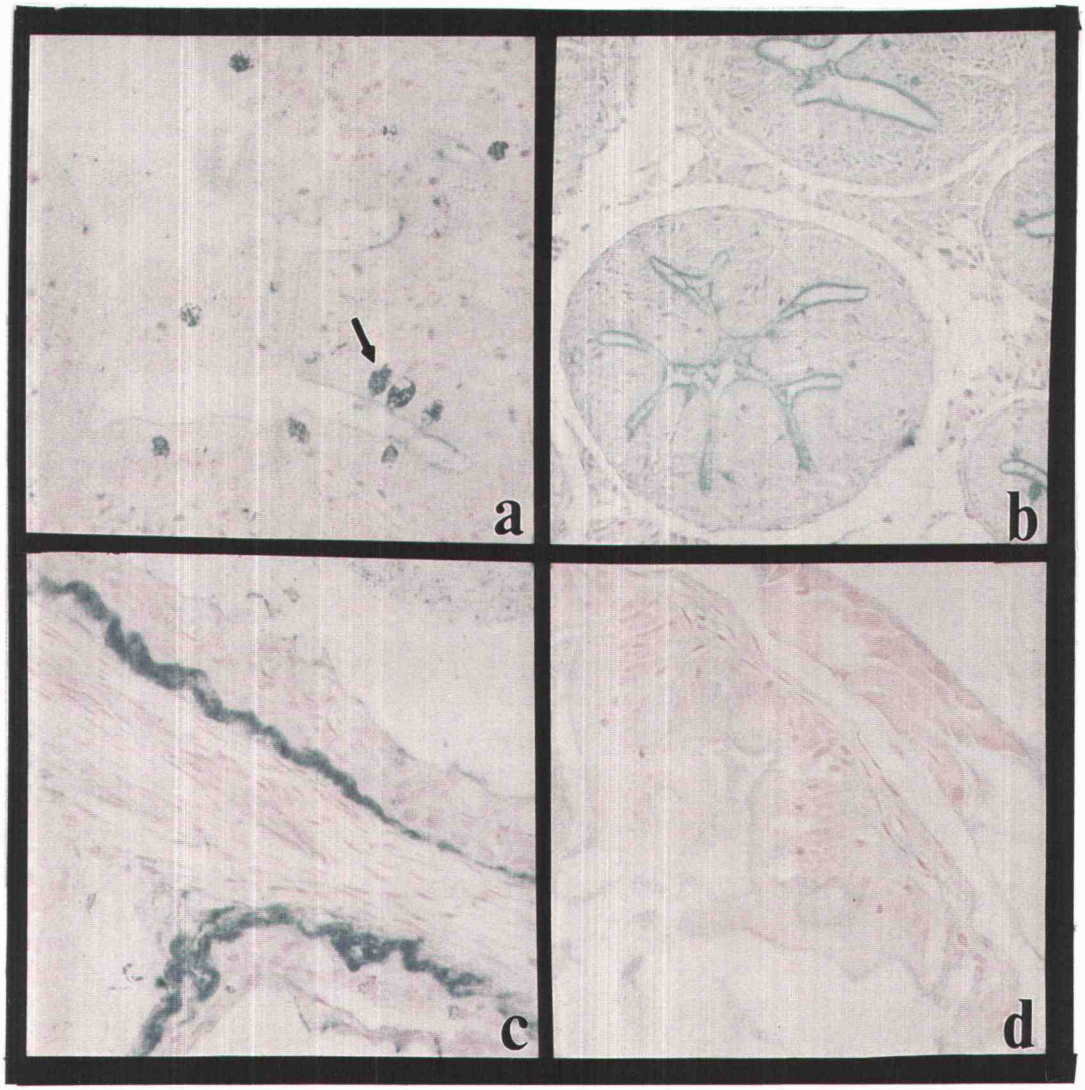

Figure 4.7 Detection of IPNV epitope AS-1 on VP2 protein by means of alkaline phosphatase immunohistochemistry (APIH) on tissue sections from experimentally infected brook trout (nuclear fast red counterstain). A positive reaction appears as a bright blue. (a) Intense positive reaction in goblet mucous cells of the intestines (arrow) (250x). (b) Positive reaction in the pyloric caeca and pancreatic tissue (100x). (c) Intense positive reaction lining the dermis/hypodermis of the epithelium (250x). (d) Negative pancreatic control tissue demonstrating the slight background obtained using the APIH procedure (250x).

the initial stages of infection (1-4 dpe), after the epizootic (62 dpe), or in the tissue of control fish.

In general, the reactions observed with the APIH were more widespread in infected tissue, with fewer foci observed (Figures 4.7b and c). However, background levels were also much higher using the APIH procedure (Figure 4.7d).

Discussion

Positive reactions using either the *in situ* hybridization or APIH procedure were variable among fish exposed to any of the virus isolates in the 10 days post-exposure for the following tissues: intestine, kidney, spleen, brain, liver, eye, cartilage, swim bladder, and thymus. The positive reactions observed in these tissues usually occurred in individual fish. Detection of viral antigen using immunofluorescence were infrequently observed in the intestinal submucosa, liver and kidney by Swanson *et al.* (1982), while stomach, spleen, gills and skeletal muscle remained negative throughout the experiment. However, Swanson *et al.* used 12-week old brook trout that had been inoculated by intraperitoneal injection, where either factor might play a large role in altering the course of the virus distribution. Additionally, the sampled fish were cut crosswise into three or four sections, which might limit the number and extent of tissue that could be observed. Yamamoto and Ke (1991) found relatively high virus titers (10^5 PFU/ml) in the gills, fins, skin, spleen, pyloric caeca, and kidney of artificially infected brook trout fingerlings. Tissue distribution of viral antigen was extensive in adult carrier fish when tested by immunoperodixase using polyclonal Ab (Reno, 1988).

The brain of infected fish infrequently displayed a positive reaction, by both *in situ* hybridization and APIH, usually in the cerebellar valvula and the cerebellum. Even more rarely a positive reaction was observed in the mensencephalon or optic lobe. Although it wasn't directly correlated, it seems possible that a positive reaction in the

brain could indicate those fish who were displaying whirling signs, due to a loss of equilibrium or vision impairment.

The virus appeared to enter the fish through the skin and muscle, possibly via the lateral line, as well as through the oral tract, gaining entry into the digestive system. Brook trout fry that had been inoculated by intraperitoneal injection (Swanson *et al.*, 1982) displayed a consistent negative reaction in the tissue of the stomach and skeletal muscle, further suggesting that these are entry routes for transmission via water contact or ingestion. Despite the suggestion that initial IPNV infection occurs at the gill surface (Smail and Munro, 1989), IPNV was not detected in or on the gills in the early stages of infection, by either *in situ* hybridization or APIH. Just prior to and during the epizootic (4-10 dpe), focal reactions using either assay were observed in what appeared to be fibroblasts within the secondary gill lamellae, as well as connective tissue located in the gill arch. However, at this time, identical focal reactions were observed in tissue throughout the fish, indicating that this was not a route of entry, but the result of a disseminated infection.

Virus appeared to become relatively dispersed thoughout the fish, displaying a distinct preference for pancreatic tissue, but also infecting individual cells or sites in other tissues. There was also a definite preference observed for what appeared to be fibroblasts in the dermal and hypodermal layers of tissue found throughout the fish, but particularly in the fins. Yamamoto and Ke (1991) found high virus titers in the fins of infected brook trout fingerlings, after infection by immersion. They suggest that viral replication in the epidermal tissues of susceptible fish establishes early in the infectious cycle, then persists during the acute stages of infection. It is a possibility that this a mechanism for site-specific dispersal of the virus. It is noted by Ellis *et al.* (1989) that the vascular hypodermis is a frequent site of developing infectious processes.

Differences among the virus isolates, as indicated by both *in situ* hybridization and APIH, include the fact that the virus isolate CSF 035-85 appeared to infect fish tissue at a faster rate than the other two isolates, producing small intense areas of focal replication in a shorter period of time (3 dpe), compared to the other two isolates, 91-137 and 91-114, which produced the areas of focal replication at 4 dpe and 6 dpe,

respectively. Virus isolate 91-114 took the longest time to establish a substantial infection, and the observed reactions caused by this isolate were generally lower in intensity initially than for the other two isolates.

From Figures 4.4, 4.5, and 4.6, using *in situ* hybridization, it is interesting to note that the day immediately preceding the appearance of the positive foci for fish exposed to each virus isolate there appears to be a decrease in the intensity and/or number of positive tissues. Possibly this indicates a stage in the viral replication when the VP2 section of the genome is not available for probe binding or the viral genome is in the middle of being replicated, followed by a sudden increase of virus and/or virus genome, resulting in the appearance of numerous locations with intense positive foci visible.

By the end of the experiment, 62 dpe, the virus had almost completely cleared from the fish, as evidenced by the limited positive reactions observed using either *in situ* hybridization or APIH. Small positive focal areas were observed in tissue of the pancreas, pyloric caeca, kidney, muscle, and skin, suggesting that limited replication of the virus continues in these tissues over time, sustaining the carrier state of infected fish.

The strong positive reaction observed in the goblet mucous cells of the gastrointestinal tract and skin using APIH, that was barely visible using *in situ* hybridization, points to a large accumulation of viral protein in these cells. It is unlikely that the reaction was due to non-specific binding or a tissue alkaline phosphatase response since the reaction was not seen throughout the infection period, it was not observed in the control tissue, and the tissue sections were treated with acetic acid prior to staining, to eliminate any tissue-associated alkaline phosphatase activity. The two most likely explanations are: intact virus about to be released, whose viral genome is somehow inaccessible to the DNA probe, or viral proteins, unassembled into virions or dissociated following assembly. If the reaction is due to intact virus, it is possible that the proteinase K treatment during the *in situ* hybridization assay caused a loss of stability that led to the disintegration of the RNA genome, before binding of the probe could occur.

This research provided an opportunity for systematic comparison between two IPNV detection assays: *in situ* hybridization, which utilizes labeled IPNV cDNA to bind to viral RNA, and APIH, which uses an IPNV monoclonal antibody to bind to viral

protein or antigen. Both procedures were successful in tracking the route of the IPN virus through cells and tissue of infected fish. It appeared that the APIH assay might have more substance to react with than the *in situ* hybridization assay, thus resulting in a more widespread positive reaction. This is probably due to the fact that the viral proteins are produced in much larger quantities than the viral genome. However, this benefit was offset by the increased background that was observed using the APIH assay. The *in situ* hybridization assay was found to produce results in tissue sections that were relatively background-free, yet with positive reactions that were clear and easy to decipher. However, given the similarities obtained in results between the assays and the relative ease in performing each assay, selecting an appropriate assay may simply be a matter of personal preference and familiarity with the required reagents. Both assays appeared to detect IPNV products (either genomic material or antigens).

Acknowledgments

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CHAPTER 5

SURVIVAL OF BROOK TROUT (*SALVELINUS FONTINALIS*) FRY EXHIBITING CLINICAL SIGNS OF INFECTIOUS PANCREATIC NECROSIS DISEASE

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Abstract

Five hundred brook trout (*Salvelinus fontinalis*) fry were experimentally infected with a virulent isolate of infectious pancreatic necrosis virus (IPNV). The purpose of the study was to determine the maximum time before death, for fish exhibiting clinical signs of IPN disease. Each day, mortalities were removed and recorded. Fish exhibiting obvious signs of IPN disease indicating a terminal conclusion were removed to a separate tank. The recovery of any fish in this tank was noted and tabulated for a period of 6 weeks. Fish were sampled representing "recovered" fish (showed signs of IPNV that disappeared at some point), mortalities (died while exhibiting signs of IPNV), moribund fish (actively showing signs of IPNV) and "asymptomatic" fish (never showed signs of IPNV). Half the fish sampled were used to determine virus titer, while the other half were embedded and sectioned for histopathology and *in situ* hybridization, using a non-radioactively labeled cDNA probe to IPNV RNA. Very few obvious differences were observed between the different groups of fish sampled. Mortalities and moribund fish appeared to have no sloughed cells in the lumen of the intestine, however, little or no unaffected pancreatic acinar tissue was observed. Both "uninfected" and "recovered" fish had large quantities of sloughed cells in the lumen of the intestine and, while necrosis was evident in the pancreas, also had some areas of unaffected pancreatic tissue remaining. Only 2.5% of fish that exhibited clinical signs of IPN disease demonstrated a recovery period, which lasted up to 2 weeks. However, brook trout fry that originally displayed signs of IPN disease all eventually succumbed and died.

Introduction

Infectious pancreatic necrosis virus (IPNV) can cause lethal disease in many aquaculture species worldwide, with the most devastating effects occurring in salmon and trout. It mainly affects young salmonids, from first feeding to approximately 6 months of age.

The disease was first described by M'Gonigle in 1941, who observed whirling behavior and high mortality in brook trout (*Salvelinus fontinalis*) fry. Mortality can vary markedly, depending upon the strain of IPNV causing the infection, however, several strains can cause upwards of 90% mortality (Wolf, 1988). The clinical signs of IPNV infection other than whirling behavior include: prostration on the bottom of the tank, overall darkening, abdominal distention, hemorrhage of ventral areas/base of fins, and castlike excretions from the vent (Wolf, 1988).

It has been generally assumed that once a fish becomes infected with IPNV and displays clinical signs of the disease, death is imminent. Wolf (1966) states that death usually occurs within 1-2 hours of obvious clinical signs of disease. However, preliminary data obtained during a mortality study of three different Buhl isolates of IPNV indicated that recovery might be possible, even after the fish became markedly moribund in appearance. For this reason, a larger study was initiated, with the results being reported here. Results demonstrate that the recovery observed is temporary, lasting up to 2 weeks. However, fingerling brook trout that originally displayed signs of IPNV all eventually succumbed and died.

Material and Methods

Cells and Virus

The Buhl strain CSF 035-85 of IPNV (American Type Culture Collection catalog #VR-890) was originally obtained as a generous gift from Dr. Scott LaPatra of Clear Springs Trout Co. in Idaho. The virus was propagated in the chinook salmon embryo (CHSE-214) cell line (Lannan *et al.*, 1984), using techniques previously described (Caswell-Reno *et al.*, 1986). Previous experiments have shown this isolate to cause 90-99% mortality in brook trout fry (Bruslind and Reno, Chapter 3 of this thesis; J. Maret, personal communication). Virus titer was determined as tissue culture infectious dose,

50% endpoint per milliliter ($TCID_{50}$/ml), calculated using the method of Spearman-Karber.

Experimental Animals

Brook trout (*Salvelinus fontinalis*) were obtained as swim-up fry (0.07 g mean wt.) from the Oregon Department of Fish and Wildlife, Wizard Falls Fish Hatchery, Camp Sherman, OR. No IPNV has been detected at this facility since 1975 (C. Banner, personal communication). The fish were held at the Oregon State University Salmon Disease Laboratory (SDL) facility in Corvallis, OR. Fish used in the experiment were approximately 0.7 g in size.

Experimental Exposure of Fish to IPNV

Five hundred brook trout fry were placed into 20 L of water at 10°C. Virus was diluted in 20 ml of Hepes-buffered minimum essential medium (HMEM) to give a final tank concentration of 10^5 $TCID_{50}$/ml (McAllister and Owens, 1986). Control fish were immersed in 20 L of water to which 20 ml of HMEM only had been added. The fish were held for 5 hours under static flow conditions and supplied with a high volume of air to maintain oxygen levels. After the immersion period, the water in the tanks was replaced with 100 L of virus-free water, the air removed, and the tank water allowed to circulate as usual. All tanks at the SDL were connected to a common flow-through system, with an approximate flow rate of 8.8 L/min. Effluent was discharged through a chlorination system at a concentration of 2.8 ppm into settling ponds, and ultimately to the Willamette River.

Daily Routine

Each day, fish which had died were removed and recorded. Fish exhibiting obvious signs of IPN disease were removed to a separate tank. Mortalities from the second tank were removed and recorded as well. The recovery of any fish in the second tank was noted and tabulated. The experiment was concluded at day 42 post-exposure (dpe), and mortality at this point was reported as percent cumulative mortality. Live fish that had been sampled during the course of the experiment were not included in the cumulative mortality calculations.

Sampling Schedule

Five dead fish were sampled on day 6 of the experiment and pooled, in order to determine viral titer. Fish sampled on day 11 represented different groups: "recovered" fish (showed signs of IPNV that disappeared), mortalities (died while exhibiting signs of IPNV), moribund fish (actively showing signs of IPNV), and "asymptomatic" fish from the original tank (never showed signs of IPNV). Four fish were sampled to represent each group. Two of the fish were used to determine virus titer, while the other two fish sampled were embedded and sectioned for histopathology and *in situ* hybridization. Fish sampled on day 42 represented "survivors," who had never showed clinical signs of IPNV infection during the course of the experiment. Nine live fish were sampled at this time and pooled in groups of three to determine viral titer. Two additional live fish were fixed in formalin for embedding and sectioning.

Tissue Fixation and Sectioning

Whole fish were preserved in 10% buffered formalin, embedded in ParaPlast Plus paraffin (Fisher Scientific, Pittsburgh, PA) and sectioned longitudinally at 5-7 um

thickness on a rotary microtome. The sections were placed on positively charged slides (Fisher Scientific). Tissue sections were rehydrated by passing the slides through a series of graded alcohols (absolute to 50%), and finally into distilled water.

Histopathology

Rehydrated tissue sections were stained with Mayer's hematoxylin, then counterstained with eosin. Sections were dehydrated in a series of graded alcohols (50% to absolute), then cleared with Slide Brite. Slides were mounted with a coverslip using Permount mounting medium. Sections were examined using normal bright field microscopy to determine the extent and location of any tissue damage.

Probe Development

IPNV cDNA of the VP2 viral capsid protein region of all three isolates was produced using the reverse transciptase polymerase chain reaction (RT-PCR). The cDNA was then used as a template for the labeling reaction. The DNA was random prime labeled with digoxigenin, using a labeling kit according to manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). Labeled probes were diluted and tested using a direct detection procedure to determine the concentration of the labeling.

In Situ Hybridization

Each tissue section was probed with 2.5 ng of digoxigenin labeled IPNV cDNA using a method similar to that described by Bruce *et al.*, 1993. A Thermolyne Amplitron^{R} II (Barnstead/Thermolyne Corporation, Dubuque, IA) with *in situ* hybridization plate accessory was used to heat the slides following addition of the probe. After the *in situ*

hybridization procedure was complete, each slide was examined using normal bright field light microscopy, for cells displaying a dark blue to purple precipitate indicating the presence of homologous IPNV RNA.

Results

Mortalities and Moribund Fish

Aside from the death of an exposed fish noted on day 2 post-exposure (dpe), the first mortalities were observed on 6 dpe, as well as the first fish displaying clinical signs of IPN disease. Affected fish were prostrate on the bottom of the tank, dark in color, and respiring rapidly; many had ascites and external hemorrhaging in the abdominal area. The number of mortalities and moribund fish peaked on 7 dpe, then rapidly dwindled in numbers. Figure 5.1 charts the progression of cumulative mortalities and moribund fish over the entire course of the experiment (42 days). At 42 dpe, the total cumulative mortality for the infected fish was 92%, while the control fish had 2% total cumulative mortality with no signs of IPN disease exhibited by control fish at any time. Out of the 92% of fish that died, 56% exhibited clinical signs of IPN disease some time preceeding death. The remaining 36% died without exhibiting overt clinical signs of IPN disease.

On 8 dpe, 6 of the fish "recovered" which exhibited clinical signs of IPN disease. An additional fish "recovered" on 10 dpe, bringing the total to 7 out of 279 moribund fish, which represented 1.4% of the total number of fish originally infected. All of these fish became lighter in color, hemorrhaging and ascites disappeared, and the fish swam around the tank in a normal fashion. No whirling characteristic of IPN disease was observed for these "recovered" fish. However, while physically appearing normal, these fish were not observed to consume food at any time.

Four of the "recovered" fish were sampled on 11 dpe for determining virus titer and embedding. One "recovered" fish died on 13 dpe, one on 22 dpe, and the final fish

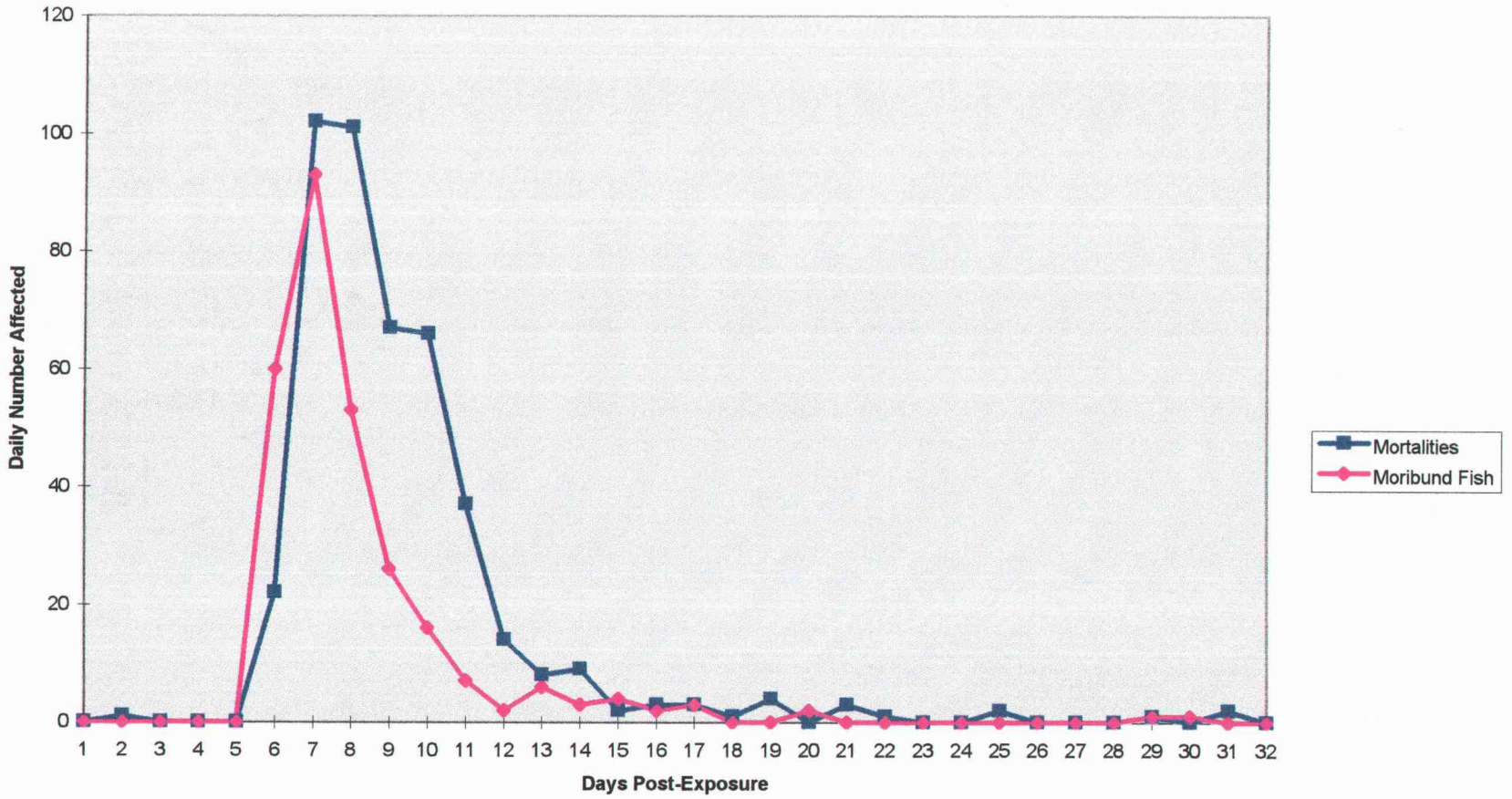

Figure 5.1 Daily mortality and moribund fish of brook trout experimentally infected with IPNV isolate CSF 035-85. Five hundred fish were infected with a challenge dose of 105 TCID50/ml. The daily affected fish (dead or moribund) are shown on the ordinate and plotted against days post-exposure. Moribund fish were those observed to display clinical signs of IPN disease.

died on 25 dpe. The fish from 22 dpe was used to determine virus titer, while the fish from 25 dpe was fixed for embedding and sectioning.

Virus Titer

Mortalities sampled on 6 dpe demonstrated a virus titer of $10^{10.2}$ $TCID_{50}/g$ (5 pooled fish), while mortalities sampled on 11 dpe demonstrated a virus titer of $10^{6.45}$ $TCID_{50}/g$ (5 pooled fish). Two moribund fish displaying clinical signs of disease that were sampled on 11 dpe both had virus titers of $10^{6.7}$ $TCID_{50}/g$. The two "asymptomatic" fish sampled on 11 dpe demonstrated virus titers of $10^{6.45}$ and $10^{8.7}$ $TCID_{50}/g$, while the "survivor" fish sampled had virus titers of $10^{6.2}$ and $10^{5.2}$ $TCID_{50}/g$. Statistically, there was no significant difference in virus titer among the different groups of fish sampled on 11 dpe (ANOVA $F_7=1.21$, $p=0.41$).

The "survivor" fish that died on 22 dpe had a virus titer of $10^{5.2}$ $TCID_{50}/g$. "Asymptomatic" fish sampled at the conclusion of the experiment on 42 dpe had virus titers of $10^{5.7}$, $10^{5.45}$ and $10^{5.2}$ $TCID_{50}/g$ (3 pooled fish each).

Histopathology

The four groups of fish sampled on 11 dpe represented: "recovered" fish (showed signs of IPN disease that disappeared), mortalities (died while exhibiting signs of IPN disease), moribund fish (actively showing signs of IPN disease), and "asymptomatic" fish (never showed signs of IPN disease). When tissue sections of these fish were examined microscopically, all displayed classical signs of IPN disease. Massive cellular disruption was characterized by nuclear pyknosis and karyorhexis. Complete breakdown of regions of the pancreatic acinar cells was evident, as well as extensive areas of necrosis. The "asymptomatic" and "recovered" fish were not observed to have regions of hemorrhaging in the abdomen and had copious amount of sloughed cells in the lumen of the intestine, unlike the mortalities and moribund fish, which had extensive hemorrhaging and little in

the lumen of the intestine. Additionally, the mortalities and moribund fish had little or no normal pancreatic tissue remaining at this time, while the "asymptomatic" fish had a moderate amount of unaffected pancreas. The "recovered" fish had some small areas of unaffected pancreas, although it was obvious that large areas of the pancreas were diseased and subsequently destroyed.

Tissue sections of the "survivor" fish that died on 25 dpe showed massive necrosis of the pancreas, both acinar and islet cells (Figure 5.2a). There were virtually no intact pancreatic cells remaining. The remaining necrotic tissue had infiltration with macrophages and erythrocytes. No sloughed cells were observed in the lumen of the intestine.

Fish sampled on 42 dpe, representing survivors, showed severe pancreatic lesions, with extensive areas of necrosis. However, there were also many intact cells amidst the necrosis, with a moderate number of unaltered pancreatic tissue regions remaining (Figure 5.2b). Nuclear pyknosis and karyorhexis were not observed, nor was abdominal hemorrhaging or sloughed cells in the lumen of the intestine.

In Situ Hybridization

The results obtained using *in situ* hybridization to detect viral RNA in the tissue sections of infected fish are reported here. Positive reactions were detected in numerous organs and tissues of infected fish, with minor variations among the different groups of fish sampled.

The reactions observed in the tissue of moribund fish and mortalities sampled on 11 dpe were very similar, with moderately positive reactions observed in locations throughout the fish. Most of the reactions observed were focal in appearance, with the strongest reactions occurring in the spleen, muscle, epidermis, and fibroblast cells. Other infected tissue included the gills, kidney, heart, brain, swim bladder, as well as the gastrointestinal tract. A positive reaction was consistently observed in the pancreas, however, it was relatively light in appearance.

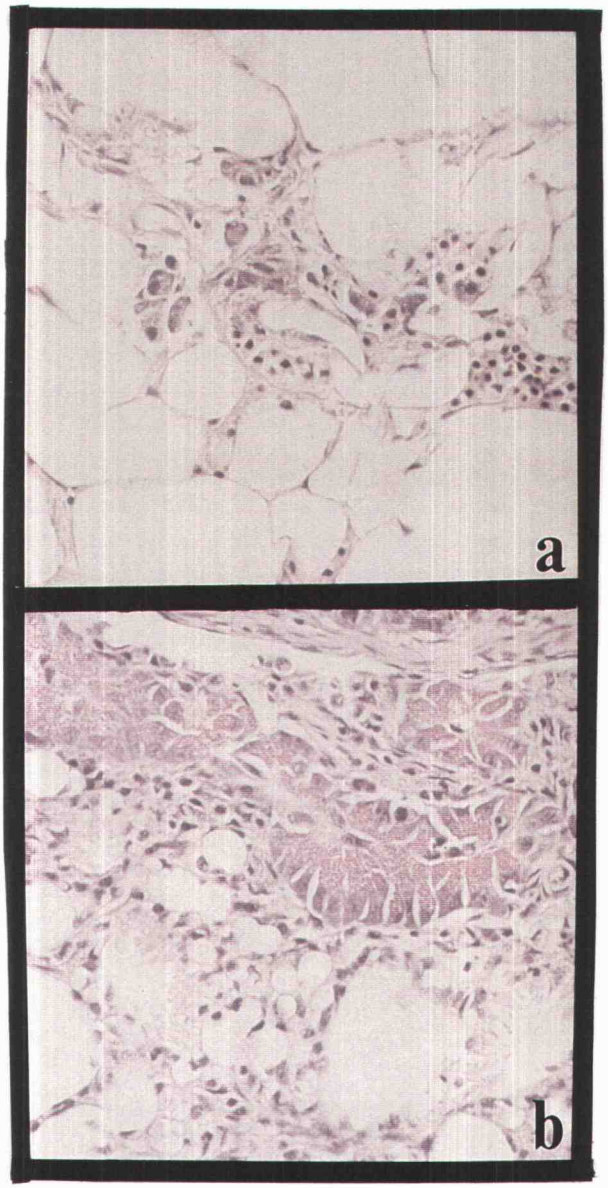

Figure 5.2 Histopathology of tissue sections from brook trout fry infected with IPNV. Sections were stained with Mayer's hematoxyling and eosin (H&E). (a) Pancreatic tissue of "survivor" fish that died at 25 dpe, showing massive necrosis with no normal acinar cells (250x). (b) Pancreatic tissue of "asymptomatic" fish sampled at 42 dpe, showing some areas of necrosis along with intact acinar cells (250x).

"Recovered" fish sampled on 11 dpe displayed stronger reactions than those seen in the moribund and mortalities, although the reactions were not quite as widespread. Positive reactions appeared confined primarily to the pancreas, tissue of the gastrointestinal tract (stomach, pyloric caeca, and intestine), muscle, and fibroblast cells. Positive foci were interspersed with areas of widespread positive reaction.

The tissue of the "asymptomatic" fish sampled on 11 dpe showed light to moderate positive reactions, mostly focal in appearance, with the strongest reactions observed in muscle tissue, skin, and fibroblast cells. Some involvement of organs of the gastrointestinal tract were observed, however, they appeared to be limiting.

The tissue of the "survivor" fish that died on 25 dpe showed light to moderate positive reactions in the gills, liver, pancreas, kidney, muscle and epidermal skin (Figure 5.3a). The reactions were focal in appearance, affecting limited numbers of cells in the infected tissue.

The positive reactions seen in tissue of the "asymptomatic" fish sampled on 42 dpe were limited to a few foci in the liver, pancreas, kidney, stomach, intestine, muscle and skin (Figure 5.3b). Although the reactions were relatively strong, based on intensity, they appeared to involve only a few cells in limited areas of the affected tissue. The majority of the tissue appeared to be uninfected.

Discussion

The observed cumulative mortality of 92% demonstrated that CSF 035-85 is one of the most virulent isolates of IPNV. However, there was no significant difference in the viral titers of fish that had died from the disease, fish that were exhibiting clinical signs of the disease, exposed fish that appeared to be healthy, and fish that exhibited clinical signs of the disease before temporarily recovering. This indicates that quantity of virus does not account for the different effects seen in infected fish.

Histopathological examination of tissue sections of infected fish indicated that

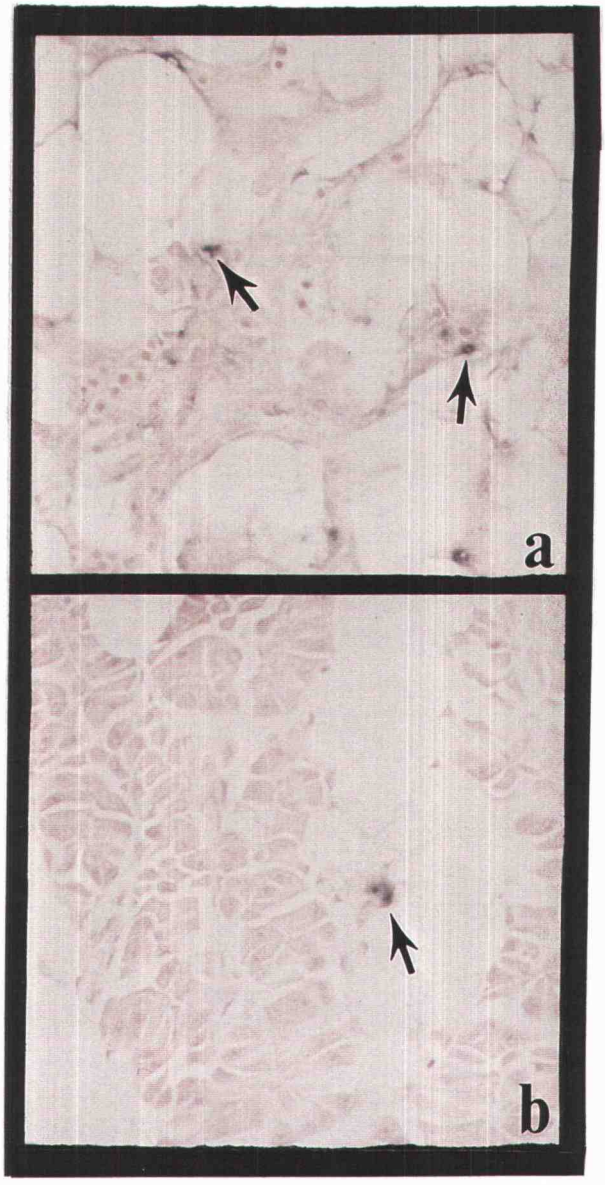

Figure 5.3 Detection of IPNV viral RNA by means of *in situ* hybridization using gene probes on tissue sections from experimentally infected brook trout fry (nuclear fast red counterstain). A purple precipitate indicates a positive reaction. (a) Pancreatic tissue of "survivor" fish that died at 25 dpe, showing a few positive focal reactions (arros) (250x). (b) Pancreatic tissue of "asymptomatic" fish sampled at 42 dpe, showing one positive focal reaction (250x).

fish which had died from IPN disease and moribund fish had little or no healthy pancreatic tissue remaining and probably suffered from complete pancreatic failure. The relatively light positive reaction observed in the pancreas, using *in situ* hybridization, was probably due to the fact that there was little pancreatic tissue remaining for the virus to infect. The pancreatic tissue of "recovered" fish and apparently healthy fish ("asymptomatic") was affected to variable degrees, but there was always some unaffected tissue observed, allowing pancreatic function to continue. Swanson *et al.* (1982) observed widespread pancreatic lesions in brook trout fry following intraperitoneal inoculation with IPNV; however, there were no mortalities. He suggested that the lesions would not critically impair pancreatic function, if intact acinar cells still remained.

"Recovered" fish and "uninfected" fish both had large amounts of sloughed cells visible in the lumen of the intestine, while the mortalities and the moribund fish did not display this characteristic. It is possible that this is due to fact that mortalities and moribund fish were in the last stage of the infection and that few cells remained in the intestine and pyloric caeca to slough off. The other two groups of fish were still actively fighting the infection, with intestinal sloughing being both a pathological feature as well as a defense mechanism of the host. It appeared that the main difference between the "uninfected" fish who survived and the "recovered" fish that eventually succumbed to the disease is the fact that the recovered fish had very limited amounts of unaffected pancreatic tissue remaining. Somehow these fish managed to rally their systems and temporarily halt the virus destruction; however, their pancreas appeared to have suffered too much damage at this point to insure survival.

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CHAPTER 6

SUMMARY

Three closely related isolates of infectious pancreatic necrosis virus (IPNV) belonging to the A_1 serotype were studied, in order to provide information into the nature of variation in the virulence of IPN viruses.

In the first experiment, groups of brook trout fry (*Salvelinus fontinalis*) were experimentally infected with the three isolates by immersion. Cumulative mortality data demonstrated that the three IPNV isolates selected had different virulence levels in brook trout fry, with two virus isolates, 91-114 and 91-137, being significantly less virulent than the virus isolate CSF 035-85. Virus titers determined for 10 days post-exposure demonstrated that there was not a significant difference among isolates. Virus titers were then correlated with fish weight to determine statistical significance. Before and during the epizootic, a positive correlation was found between virus titer and fish weight, for all three isolates. After the epizootic (62 dpe), fish weight was found to negatively correlate to virus titer for the two least virulent isolates (91-137 and 91-114), while there was no correlation for the most virulent isolate, CSF 035-85.

The nucleotide sequence of the IPNV capsid proteins VP2 and VP3 was determined, in order to elucidate a possible molecular basis for the difference in virulence among isolates. Initial sequencing results demonstrated sequence homology for the viral capsid protein VP3, while slight differences among isolates were observed for the viral capsid protein VP2. The VP2 region was sequenced for each virus isolate at three times: before being introduced into the fish, during the epizootic, and 2 months after exposure, in order to determine if major changes existed in the VP2 region that might account for the differences in virulences. Two amino acid changes between the less virulent isolates, 91-137 and 91-114, and the most virulent isolate CSF 035-85 occurred at residues 217 and 288 in the VP2 region, distinguishing the least virulent isolates and the most virulent isolate. It is proposed that these amino acid differences might account for the disparity in expressed virulence for these particular IPNV isolates.

Samples of live whole fish exhibiting slight clinical signs of disease were collected before, during, and after the epizootic. Collected samples were fixed, embedded whole, and sectioned using a microtome. The progression of the IPNV virus was tracked through the tissues for days 1-10 post-infection and at the end of the experiment (62 dpe) using histopathology, alkaline phosphatase immunohistochemistry (APIH), and *in situ* hybridization. Histopathology was performed by examining tissue sections stained with hematoxylin and eosin (H&E), and demonstrated that histopathology signs were limited primarily to the pancreas and pyloric caeca of infected fish.

APIH was performed using a monoclonal antibody (AS-1) specific to the VP2 viral capsid protein of IPNV, while the *in situ* hybridization was performed using cDNA reverse transcribed and amplified from the VP2 region, which was then randomly labeled with digoxigenin. Immunohistochemistry and *in situ* hybridization reactions, which paralleled each other, were observed in tissue throughout infected fish. The tissue most prominently affected was pancreas, as well as what appeared to be fibroblasts found in the dermis/hypodermis of tissue layers throughout the fish, but especially in fins. Based on observed results, an IPNV infection appeared to be established in the fish by two routes: the muscle, most likely by entering the lateral line through contact with the water, and from the oral region into the gastrointestinal tract by ingestion.

Assay results between the two procedures were comparable, with only one reaction being observed with the APIH procedure that was barely observed using in situ hybridization. Using APIH, a very strong positive reaction was observed in the goblet mucous cells lining the intestinal mucosa and the epithelial skin. This points to possible accumulation of virus and/or viral proteins in these cells. It was also determined that the APIH procedure has higher background levels than the *in situ* hybridization.

Preliminary data obtained during the first study of the three different isolates of IPNV indicated that recovery of infected fish might be possible, even after the fish became moribund in appearance, contrary to previous statements (Wolf, 1966). For this reason, a larger study was initiated. An artificial infection was initiated, using susceptible brook trout fry approximately 1 g in size. Severely moribund fish exhibiting obvious signs of IPNV were removed to a separate tank. The recovery of any fish in this tank was

noted and tabulated. Fish were sampled representing "recovered" fish (showed signs of IPNV that disappeared at some point), mortalities (died while exhibiting signs of IPNV), moribund fish (actively showing signs of IPNV) and "asymptomatic" (never showed signs of IPNV). Half the fish sampled were used to determine viral titer, while the other half were embedded and sectioned for histopathology and *in situ* hybridization, using a non-radioactively labeled cDNA probe to IPNV RNA. Very few obvious differences were observed among the different groups of fish sampled. Mortalities and moribund fish appeared to have no sloughed cells in the lumen of the intestine, however, little or no unaffected pancreatic acinar tissue was observed. Both "uninfected" and "recovered" fish had large quantities of sloughed cells in the lumen of the intestine and, while necrosis was evident in the pancreas, also had some areas of unaffected pancreatic tissue remaining. Results demonstrate that the recovery observed is temporary, lasting up to 2 weeks. However, fingerling brook trout that originally displayed signs of IPNV all eventually succumbed and died, which may explain the low grade mortalities that occur after the peak of the epizootic has been reached. This may occur rather than the secondary infection of a subpopulation of fish which had not been infected the initial epizootic.

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APPENDIX
PROTOCOLS FOR THESIS ASSAYS

Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)
Protocols for IPNV Infected Samples

TRIzol Extraction of Nucleic Acid from Fish Homogenates

1. Add 100 ul of infected sample to 1 ml of TRIzol reagent (GibcoBRL). Mix and leave for 5 min, room temperature (RT) to complete disassociation of any nuceloprotein complexes.

2. Add 200 ul of chloroform, shake for 15 sec, then incubate at RT for 3 min.

3. Microfuge at 11,500 g for 15 min, 4°C. Remove the aqueous (top) layer to a new tube and add 500 ul of isopropanol.

4. Incubate samples for 10 min, RT. Microfuge at 11,500 g for 10 min, 4°C.

5. Discard the supernatant and wash the resulting pellet with 1 ml of 75% ethanol. Microfuge at 7,500 g for 5 min, 4°C.

6. Pour off the ethanol and let the pellet air-dry for 10 min, RT.

7. Resuspend the pellet in 10 ul of DNase-, RNase-free water, then place at 55°C for 10 min to improve dissolution of the pellet.

Reverse Transcription (RT)

1. Dilute extracted RNA 1:10 in water, heat at 95°C for 5 min, microfuge briefly, then place on ice for 2 min.

2. Add the following reagents together: 1 ul of diluted viral RNA, 1 ul (1.25 mM) 25mM magnesium chloride ($MgCl_2$), 1 ul (0.25x) 5x buffer (Promega), 1 ul (33 units) RNasin (Promega), 1 ul (200 units) reverse transcriptase (Promega), 1ul (55 pmol) 3' primer, 1 ul (55 pmol) 5' primer, 1 ul (1mM each dATP, dCTP, dGTP, dTTP) deoxynucleotides (dNTPs), 12 ul water. Final $MgCl_2$ concentration for the reaction is 2.0 mM. Mix, microfuge briefly, then place at 37°C for 1 h.

3. Heat the samples at 95°C for 3 min, microfuge briefly, then place on ice for 2 min.

4. Add 1 additional ul (200 units) of reverse transcriptase to each reaction, mix, microfuge briefly, then place at 37°C for 1 h.

5. Proceed immediately with polymerase chain reaction (PCR).

Polymerase Chain Reaction (PCR)

1. Heat the 20 ul reaction from the RT at 95°C for 5 min, microfuge briefly, then place on ice.

2. Add the following reagents: 8 ul 25mM $MgCl_2$, 10 ul 10x PCR buffer (Promega), 1 ul (55 pmol) 3' primer, 1 ul (55 pmol) 5' primer, 0.5 (2.5 units) Taq polymerase (Promega), 59.5 ul water. Mix and microfuge briefly.

3. Place the samples in a Thermolyne Temp-tronic^R (Barnstead/Thermolyne Corporation) for 36 cycles with the following program: 94°C - 1 min, 58°C - 1 min, 72°C - 2 min. Hold samples at 4°C, until samples can be retrieved.

4. Run 5 ul of each sample on a 1% agarose gel made with TAE (40 mM Tris, 20mM acetic acid, 2 mM EDTA), with ethidium bromide (EtBr) added, and electrophorese at 75 V for 1.5 h. Visualize gel with a UV light box.

QIAquick Purification of PCR Products

1. Add 500 ul of PB buffer (Qiagen) to each PCR reaction.

2. Apply the sample to a QIAquick spin column (Qiagen), place in a 2 ml collection tube, and microfuge for 30-60 sec.

3. Discard the flow-through. Add 750 ul of PE buffer (Qiagen) to the column, and microfuge the column for 30-60 sec.

4. Discard the flow-through and microfuge the column for an additional minute.

5. Place the column in a clean 1.5 ml microfuge tube. Add 50 ul of DNase-, RNase-free water and microfuge the column for 1 min. Place the sample at -70°C for storage.

IPNV Detection in Paraffin Embedded Fish Tissues using Alkaline Phosphatase Immunohistochemistry or Non-Radioactive *In Situ* Hybridization

Tissue Fixation and Sectioning

1. Preserve whole fish in 10% buffered formalin for at least 48 h, rinse with distilled water for 8 h, then place in 70% ethanol until ready to process.

2. Place whole fish in tissue cassettes. Process fish by using a Tissue-Tek^{R} II tissue processor, which dehydrates tissue through a series of alcohols, followed by xylene, and paraffin.

3. Embed preserved fish in ParaPlast Plus paraffin (Fisher Scientific) and section at 5-7 um thickness on a rotary microtome.

4. Place sections on positively charged microscope slides (Fisher Scientific).

Histology

1. Rehydrate tissue sections through a series of graded alcohol, and finally into distilled water.

2. Stain the tissue sections with Mayers hematoxylin for 4-6 min, then placed in running water for 20 min to blue the nuclei.

3. Counterstain the tissue sections with eosin for 2 min.

4. Dehydrate by passing the slides through a series of graded alcohols (50% to absolute) and finally into clearing agent, 2x for 15 min each.

5. Mount slides with a coverslip using Permount mounting medium. Examine tissue sections, using normal bright field microscopy, to determine the extent and location of any tissue damage.

Immunohistochemistry

1. Embed tissue in paraffin and cut sections that are 4 um or less in thickness. Put sections onto positively charged microscope slides.

2. Rehydrate tissue as follows:

Clearing agent	2x	5 min each
100% alcohol	1x	5 min
95% alcohol	2x	5 min each

70% alcohol	1x	5 min

3. Wash the slides for 10 min in running water.

4. Dip slides for 15 seconds in 20% glacial acetic acid at 4°C. Rinse with water.

5. Equilibrate slides in phosphate buffered saline (PBS), pH 7.4 for 20 min, RT.

6. Block tissue with 2% normal serum for 20 min, RT. Blot excess block from sections.

7. Block tissue with 5% milk in PBS for 40 min, RT. Blot excess block from sections.

8. Incubate with primary antibody for 1 h, RT.

9. Rinse slide with PBS, 2 x 5 min each, RT.

10. Incubate with biotinylated secondary antibody, diluted in PBS, for 40 min, RT.

11. Rinse slide with PBS, 2 x 5 min each, RT.

12. Incubate with Avidin-Biotin Complex (ABC) solution for 30 min, RT.

13. Rinse slide with PBS, 2 x 5 min each, RT.

14. Equilibrate sections with 100mM Tris, pH 8.2, for 3 min, RT.

15. Incubate with Vector^{R} Blue Substrate solution for 40 min at 37°C, in the dark.

16. Rinse slides with tap water. Counterstain tissues with Vector^{R} Nuclear Fast Red for 30 sec.

17. Place slides in running water for 10 min.

18. Dehydrate the slides as follows:

70% alcohol	1x	10 dips
95% alcohol	2x	10 dips each
100% alcohol	2x	10 dips each
Clearing agent	2x	10 dips each

19. Mount with coverslip and mounting medium (Permount). Examine sections under bright field looking for bright blue precipitate.

REAGENTS

Phosphate buffered saline (PBS)

NaCl	8 g
KH_2PO_4	0.2 g
Na_2HO_4	1.15 g
KCl	0.2 g
dd H_2O	to 1 L

pH to 7.4 with NaOH; autoclave to sterilize, store at RT.

Blocking serum (prepare just prior to use)

Blocking serum (VectastainR kit)	3 drops
PBS	10 ml

5% Milk block

Nonfat dry powdered milk	5 g
PBS	95 ml

Store at 4°C.

Biotinylated secondary antibody (prepare just prior to use)

Biotinylated anti-mouse antibody (Vectastain)	1 drop
PBS	10 ml

Mix well by shaking before use.

Avidin-biotin complex (ABC) solution (let sit at RT for 30 min before use)

Solution A (Vectastain)	1 drop
Solution B (Vectastain)	1 drop
PBS	5 ml

Mix well by shaking after each solution is added.

100 mM Tris

100 mM Tris base
dd H_20
pH to 8.2; store at 4°C.

Blue phosphate substrate (prepare just prior to use)

Levamisole (Vector^R)	1 drop
Solution #1 (Vectastain)	2 drops
Solution #2 (Vectastain)	2 drops
Solution #3 (Vectastain)	2 drops
100 mM Tris	5 ml

Mix well by shaking after each solution is added.

Labeling of IPNV DNA with DIG

1. Use 300 ng of purified template in a volume of 15 ul or less for each labeling reaction. Bring the volume up to 15 ul, if necessary, with ddH_20.

2. Denature the DNA for 10 min in a boiling water bath, then chill the DNA on ice for 3 min.

3. Add the following components: 2 ul hexanucleotide mixture, 2 ul dNTP labeling mixture, 1 ul (2 units) Klenow enzyme. Mix, microfuge briefly.

4. Incubate the mixture at 37°C overnight.

5. After incubation, add the following: 2 ul 0.2 M EDTA (pH 8.0), 2.5 ul 4 M LiCl, and 75 ul ice cold ethanol. Mix.

6. Place the solution at -20°C for 2 h or -70°C for 45 min.

7. Microfuge for 10 min, 10,000 g. Discard the supernatant and wash the pellet in 70% ethanol.

8. Let air dry for 20-30 min, RT.

9. Resuspend the pellet in 50 ul 0.1 x Tris.EDTA.

In Situ Hybridization with DIG-Labeled Probes

1. Embed tissue in paraffin and cut sections that are 4um or less in thickness. Put sections onto positively charged microscope slides.

2. Heat slides in oven for 30 min at 65°C. Rehydrate tissue as follows:

Clearing agent	2x	5 min each
100% alcohol	1x	1 min
95% alcohol	2x	10 dips each
70% alcohol	1x	5 min

3. Wash the slides for 10 min in running water.

4. Wash the slides for 10 min in PBS. Prepare proteinase K fresh at 100 ug/ml in PBS. Pipet on 1 ml per slide of proteinase K solution and incubate 15 min, 37°C, in a humid chamber.

5. Wash the slides in PBS with 0.2% glycine for 10 min at RT to inactivate the enzyme.

6. Incubate slides in 2x SSC for 10 min at RT.

7. Add 0.5 ml hybridization buffer per slide and incubate in humid chamber for 1.5 h at RT.

8. Boil the DIG-labeled probe for 10 min and quench on ice; spin briefly in the cold and keep on ice. Dilute the probe to 10 ng/ml in hybridization solution and flood tissue with 250 ul of the solution.

9. Carefully place a coverslip on top of each slide. Heat slides at 95°C for 3 min, followed by 2 min on ice. Incubate slides overnight at 37°C in a humid chamber.

10. Wash the slides as follows:

2x SSC	1 h	RT
1x SSC	1 h	RT (warm 0.5x SSC during this step)
0.5x SSC	30 min	37°C
0.5x SSC	30 min	RT

11. Wash the slides for 5 min in Buffer I, RT. Block the slides with 1 ml per slide of Buffer II. Incubate 30 min, RT.

12. Dilute the AP-DIG conjugate 1:5000 in Buffer I containing 1% normal sheep serum and 0.3% Triton X-100. Flood tissue with 500 ul of diluted conjugate and incubate in humid chamber, 45 min, RT.

13. Wash the slides in Buffer I, 2 x 15 min each, RT. Equilibrate slides in Buffer III for 5 min, RT.

14. Pipet on 500 ul of color reagent per slide and incubate in a dark, humid chamber, overnight at RT.

15. Stop the reaction by washing the slides in Buffer IV, 10 min, RT.

16. Dehydrate the slides as follows:

distilled water	1x	10 dips
70% alcohol	1x	10 dips
95% alcohol	2x	10 dips each
100% alcohol	2x	10 dips each
Clearing agent	2x	10 dips each

17. Mount with coverslip and mounting medium (Permount). Examine sections under bright field looking for dark blue to black precipitate.

REAGENTS

Phosphate buffered saline (PBS)

NaCl	16 g
KH_2PO_4	0.4 g
Na_2HO_4	2.3 g
KCl	0.4 g
dd H_2O	to 2 L

pH to 8.2 with NaOH; autoclave to sterilize, store at RT

Proteinase K (prepare fresh prior to use)

PBS	10 ml
Proteinase K	1 mg

0.2% Glycine

PBS	100 ml
Glycine	0.2 g

Filter solution, store at 4°C.

Prehybridization Buffer

4x SSC (see below)
50% Formamide
1x Denhardt's (see below)
5% Dextran sufate
0.5 mg/ml herring sperm DNA (boil 10', shear through needle)

Hybridization Buffer

Same as prehybridization buffer, without herring sperm DNA.

20x SSC Buffer

3 M NaCl
0.3 M $Na_3C_6H_5O_7 \cdot 2H_20$
dd H_20
pH to 7.0; autoclave, store at 4°C

20x Denhardt's

Bovine serum albumim (Fraction V)	0.4 g
Ficoll 400	0.4 g
Polyvinylpropyl 360	0.4 g
dd H_20	100 ml

Filter through 0.45 um filter; store at 4°C.

Buffer I

0.1 M Tris base
0.15 M NaCl
dd H_20
pH to 7.5 with Hcl; autoclave; store at 4°C.

Buffer II (blocking buffer)

Buffer I	49 ml
Normal sheep serum	1 ml
Triton X-100	0.15 ml

Store at 4°C for up to 2 months.

AP-DIG conjugate dilution buffer

Buffer I	49.5 ml
Normal sheep serum	0.5 ml
Triton X-100	0.15 ml

Store at 4°C for up to 2 months.

Buffer III

100 mM Tris base
100 mM NaCl
50 mM $MgCl_2$
dd H_20
pH to 9.5 with HCl filter through 0.45 um filter; store at 4°C.

Buffer IV

10 mM Tris base
1 mM EDTA
dd H_20
pH to 8.0 with HCl filter through 0.45 um filter; store at 4°C.

Development solution (prepare just prior to use)

Levamisole	4mg
Nitroblue tetrazolium salt (Boehringer Mannheim)	45 ul
5-bromo-4-chloro-3-indoyl phospahte, toluidinum salt (Boehringer Mannheim)	35 ul
Buffer III	10 ml