

Epizootic Haematopoietic Necrosis Virus: Epidemiology and Uncertainty

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Abstract

Epizootic haematopoietic necrosis virus (EHNV) was the first virus isolated from fish in Australia, and the first iridovirus associated with systemic infection and epizootic mortality. It is one of only five diseases of finfish that are notifiable to the Office International des Epizooties (OIE). The EHNV causes epizootic mortality in wild redfin perch (*Perca fluviatilis*) and a fatal disease of low prevalence in farmed rainbow trout (*Oncorhynchus mykiss*), while related viruses (European sheatfish virus, ESV/European catfish virus, ECV) cause epizootic mortality in sheatfish (*Silurus glanis*) and catfish (*Ictalurus melas*) in Europe. Being an indiscriminate pathogen, EHNV lacks host specificity but displays considerable variation in virulence in different species of finfish. Detection of EHNV in rainbow trout can be difficult because the mortality rate may barely rise above background levels. However, EHNV can be detected in affected fish among 'routine' mortalities and may be associated with specific antibodies in a small proportion of older fish. Sampling to detect EHNV for certification purposes should be based on examination of 'routine' mortalities rather than random samples of live fish. Further studies are needed to identify the environmental reservoir of the ranaviruses found in finfish because fish may be accidental hosts. The natural reservoir could be among the amphibians and reptiles because redfin perch, sheath fish and catfish seem to be too susceptible and rainbow trout, too resistant to account for the ecological persistence of the virus. This research will assist in the critical assessment of the role of legal and illegal trade in food fish, ornamental fish, reptiles and amphibians in the trans-border spread of these viruses.

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Introduction

Epizootic haematopoietic necrosis virus (EHNV) was the first virus isolated from fish in Australia and the first iridovirus shown to be associated with multisystemic necrosis and epizootic mortality (Langdon et al. 1986b; Langdon and Humphrey 1987; Langdon et al. 1988). It is one of only five finfish disease agents notifiable to the Office International des Epizooties (OIE) (OIE 2002). This listing followed an assessment of its significance: high virulence in certain hosts, lack of host specificity, lack of an effective treatment, high socio-economic cost of disease outbreaks, restricted geographic range and proven aetiology (Langdon et al. 1986b; Langdon 1989; Whittington et al. 1996; 1999). The OIE Reference Laboratory for EHNV is located in Australia as a joint undertaking of the Faculty of Veterinary Science at the University of Sydney and the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Australian Animal Health Laboratory (AAHL). While EHNV is unknown outside Australia, closely related viruses have emerged in Europe and collectively the iridoviruses are emerging as an important group of pathogens for cultured and wild finfish in many countries.

The key to control of EHNV and related viruses is detailed knowledge of their epidemiology. Factors such as taxonomic status, host range, reservoir hosts, infectivity, virulence, persistence in the environment, rates of transmission, sensitivity of diagnostic tests, and means of disease certification are all important. In this paper the available information is reviewed and gaps in our knowledge are highlighted. Much is yet to be discovered.

Taxonomy of EHNV and related viruses

The EHNV belongs in the genus *Ranavirus* in the family *Iridoviridae* with the type species Frog virus 3 (FV3) (Langdon et al. 1986b; Eaton et al. 1991; Hedrick et al. 1992; Hengstberger et al. 1993; Hyatt et al. 2000). Officially the family consists only of two named genera that infect mainly insects and other arthropods (*Iridovirus* and *Chloriridovirus*), and two named genera that infect vertebrates (*Ranavirus* and *Lymphocystivirus*) (Williams et al. 2000). To date no demarcation criteria have been listed for the genus *Ranavirus* and FV3 is the only listed species in the genus. Tentative species include EHNV, Bohle virus (BIV), Redwood Park virus, Regina virus and Santee-Cooper ranavirus. However, there are many other iridoviruses of lower vertebrates that do not fall into any of these genera (see

below), and there are many unassigned ranaviruses. Ranaviruses have been isolated from healthy or diseased frogs and salamanders in America, Europe and England (Wolf et al. 1968; Fijan et al. 1991; Drury et al. 1995; Zupanovic et al. 1998; Chinchar 2002). One (BIV) was isolated from diseased tadpoles of the frog *Limnodynastes ornatus* in Queensland, Australia (Speare and Smith 1992). Ranaviruses have also been recovered from reptiles (Hyatt et al. 2002). As FV3 is the only member of the genus *Ranavirus* that is recognised by the International Committee on the Taxonomy of Viruses, before any virus can be included in this genus it must be compared to FV3. Ranaviruses have large (145-162 nm), icosahedral virions, a double stranded DNA genome and like others in the family, replicate in both nucleus and cytoplasm with cytoplasmic assembly. They possess common antigens which can be detected by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence, but no effective neutralising antibodies have been produced to assist identification. To distinguish between the ranaviruses, the genome can be examined by restriction enzyme digestion, cross-hybridisation or sequencing. A critical review of iridoviral taxonomy is required.

Since the recognition of disease in Australia due to EHNV in 1986, a number of similar systemic necrotising iridovirus syndromes have been reported in farmed fish. Epizootics have occurred in France in catfish (*Ictalurus melas*) (European catfish virus, ECV), and in Germany in sheatfish (*Silurus glanis*) (European sheatfish virus, ESV) (Ahne et al. 1989; Pozet et al. 1992). A virus in turbot (*Scophthalmus maximus*) in Denmark may be in the same group (Bloch and Larsen 1993). The ESV has recently spread to Finland. The EHNV, BIV, ECV and ESV have been examined in accordance with the criteria mentioned above and are unofficially recognised as members of the genus *Ranavirus*, while FV3, EHNV, BIV and ECV/ESV appear to be distinct viruses (Hedrick et al. 1992; Mao et al. 1997; Ahne et al. 1998; Hyatt et al. 2000) although until recently there was no simple means to differentiate them. Therefore ECV and ESV are regarded as being synonymous with EHNV in guidelines developed by the Fish Diseases Commission of the OIE (OIE 2000). However, restriction analysis of the genome indicated that the European fish ranaviruses differed from EHNV and frog ranaviruses (Ahne et al. 1998; Hyatt et al. 2000). Variations in the nucleotide sequence of the major capsid protein gene (MCP) were also found (Mao et al. 1996; 1997; Hyatt et al. 2000) and complete sequencing of MCP has since led to a rapid test to differentiate these ranaviruses (Marsh et al. 2002).

Pathology

The EHNV causes distinctive systemic necrotising lesions in haematopoietic and other tissues, often with basophilic intracytoplasmic viral inclusions (Reddacliff and Whittington 1996). This pathology differentiates ranaviruses like EHNV from several other groups of iridoviruses that infect finfish and is a useful taxonomic indicator. For example, fish erythrocytic iridoviruses (piscine erythrocytic necrosis, PEN) cause inclusions in red blood cells, viruses in the *Lymphocystivirus* genus infect fibroblasts and cause dramatic cytomegally leading to visible swellings in skin, while iridoviruses from cichlids, grouper, red sea bream and other species from south and southeast Asia produce inclusion body containing cells that are sometimes mistaken for intracellular protozoa (Leibovitz and Riis 1980; Armstrong and Ferguson 1989; Inouye et al. 1992; Anderson et al. 1993; Fraser et al. 1993; Chua et al. 1994; Williams et al. 2000). Only EHNV and closely related viruses will be addressed further in this review.

Host range

Natural EHNV infections are known in two teleost species, redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) (Langdon et al. 1986b; Langdon and Humphrey 1987; Langdon et al. 1988), however, many other finfish species are susceptible to EHNV experimentally. Individuals of the following species died with systemic necrosis after bath inoculation: Macquarie perch (*Macquaria australasica*), silver perch (*Bidyanus bidyanus*), mosquito fish (*Gambusia affinis*) and mountain galaxias (*Galaxias olidus*). Murray cod (*Maccullochella peelii*), golden perch (*Macquaria ambigua*), Australian bass (*Macquaria novemaculeata*), Macquarie perch, silver perch and Atlantic salmon (*Salmo salar*) were susceptible following intraperitoneal injection of virus while cyprinids appeared to be resistant (Langdon et al. 1986b; Langdon 1989). Based on these results EHNV can be classified as an indiscriminate pathogen. Redfin perch and mosquito fish were introduced to Australia and although redfin perch are sought by sports fishermen, both are considered by ecologists to be pest species. Rainbow trout and Atlantic salmon, also imported, are commercially significant. The other susceptible species are native to Australia, found in natural freshwater ecosystems and considered vulnerable or endangered. Some of these are farmed on a small scale. EHNV therefore presents a challenge to commercial fisheries and is an ecosystem management threat. It is surprising that so little study of its distribution and effect on native fish populations has been conducted.

Geographic range

The known geographic distribution of infected hosts is shown in [figure 1](#) and the pattern of spread in New South Wales is indicated in [figure 2](#).



Figure 1. Known geographic extent of EHNv infected fish stocks. Within the endemic zone (shaded), there is a discontinuous distribution

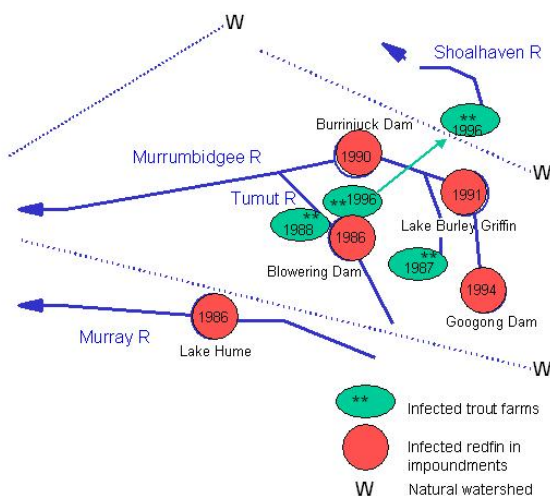


Figure 2. Known pattern of spread of EHNv in wild redfin perch and farmed rainbow trout between 1986 and 1996 in New South Wales

Rainbow trout

To date only farmed rainbow trout are known to be infected, but wild stocks, which are present in many rivers and impoundments in southeastern Australia have not been examined. Rainbow trout in Australia are farmed in open systems that draw water from rivers, dams or bores. The EHNv is known to occur on a small number of farms in the south east. Some farms within this region have remained free of the disease (Whittington et al. 1999). Tasmania and Western Australia remain free of infection in salmonids and have a program of active surveillance based on ELISA or virus isolation.

Redfin perch

Redfin perch are not farmed in Australia; the species occurs wild in many river systems and impoundments in southern latitudes. There is endemic infection of redfin perch with EHNv in southeastern Australia but there is a discontinuous distribution. Since 1986 the disease has spread progressively upstream in the Murrumbidgee River system through New South Wales and the Australian Capital Territory. Similar spread has been observed in the Murray River in South Australia (Whittington et al. 1996).

Prevalence

Rainbow trout

It was first thought that disease due to EHNv occurred mainly in young fingerlings <125 mm forklength where daily mortality of less than 0.2% and total mortality up to only 4% has been reported. However, it was later shown that hatchery fry and 1+ to 2+ grower fish may also succumb. Although infection has not yet been seen in broodstock, it is possible that fish of all ages are susceptible (Whittington et al. 1994; 1999). During outbreaks, EHNv has been detected in 60-80% of moribund or dead fish, but in only 0 to 4% of in-contact, clinically healthy fish. The 99% confidence limits for the prevalence of subclinical infection are 0-8% based on samples of 150 fish. The virus could not be found at all in surviving cohorts after an outbreak. It appears that EHNv is poorly infective but has a high case fatality rate. Anti-EHNv antibodies can be detected in grower fish but again at low prevalence (0.7%, 95% confidence limits 0.02% to 3.7%). EHNv may be present on a farm without causing suspicion because the mortality rate may not rise much above the usual background rate.

Redfin perch

The disease is recognised by spectacular epizootic mortality in fish of any age, affecting a very large proportion of the population. Typically fingerling and juvenile fish are affected in endemic areas but in newly infected areas adults have also been affected. When the disease is first recognised in an area, there is a dramatic population decline, with loss of the recreational fishery for years (Langdon et al. 1986b; Langdon and Humphrey 1987; Whittington et al. 1996).

Source of infection

Movement of infected trout fingerlings is the common means of spread of EHNW within the trout industry (Whittington et al. 1999) (Fig. 2). It is assumed that consignments of fish contain a low proportion of individuals with progressive subclinical or clinical infection, rather than carrier fish. Annual recurrence in farmed rainbow trout is likely to be due to reinfection of successive batches of fish from wild redfin perch in the same catchment (Fig. 2). Restriction endonuclease analysis of DNA from viruses recovered from rainbow trout and redfin perch in the same catchment has confirmed that the isolates are very similar (Hyatt et al. 2000). Near-complete sequence (1472 bp) for the MCP of EHNW isolates from rainbow trout and redfin perch were identical (Marsh et al. 2002). As the virus is resistant in the environment, it may also persist in sediments and on equipment and surfaces. The reservoir of infection for redfin perch is uncertain; it might be environmental, in the redfin population or in another host. This will be discussed below.

Variation in susceptibility of different host species

There are dramatic differences in the susceptibility of redfin perch and rainbow trout to EHNW (Table 1). Redfin perch are exquisitely susceptible to EHNW. Experimental bath inoculation with as few as $0.08 \text{ TCID}_{50} \cdot \text{mL}^{-1}$ was lethal, and doses too low to be detected by virus isolation in BF-2 cells can be fatal by intraperitoneal inoculation (Whittington and Reddacliff 1995). In contrast, and again in keeping with the natural pattern of disease, rainbow trout were resistant to bath exposure in $10^{2.2} \text{ TCID}_{50} \cdot \text{mL}^{-1}$ (Whittington and Reddacliff 1995), while only 1 of 7 became infected after bath inoculation for 1 hour in 10^3 TCID_{50} per ml (Langdon et al. 1988). The findings suggest that EHNW is poorly infective for rainbow trout.

Table 1. Susceptibility of the known natural hosts to EHNV

Measure	Redfin perch	Rainbow trout
Disease	Fatal multisystemic	Fatal multisystemic
Prevalence during outbreak	Very high (95%)	Very low (0-4%)*
Bath inoculation	0.08 TCID ₅₀ /ml	Very resistant
Ages affected	All	0+ to 2+
Carriers	Unlikely	Unlikely**

*99% confidence limits for subclinical infection 0 - 8%; **carrier recruitment rate <2%

Reservoir hosts

A carrier state is an inapparent infection that may lead to shedding of virus. [Langdon \(1989\)](#) thought it possible that Murray cod could act as a carrier species as infection without disease occurred after bath inoculation. It is uncertain whether a true EHNV-carrier state is possible in rainbow trout. Persistent infection with very small numbers of infectious virions was detected in one clinically healthy rainbow trout fingerling 63 days after intraperitoneal inoculation ([Whittington and Reddacliff 1995](#)). The significance of this observation is unclear because of the artificial route of infection. Virus has been detected in grower fish but as histopathological lesions consistent with EHNV were also present there was active infection rather than a carrier state ([Whittington et al. 1999](#)). There is no evidence yet that rainbow trout broodstock become infected, but too few samples have been examined to be certain; although no infection was detected, the prevalence of infection in broodstock on an infected farm could only be stated to be <20% (with 99% confidence) because of small sample size ([Whittington et al. 1994](#)). Anti-EHNV antibodies can be detected in serum from 1+ to 2+ grower fish but not 0+ fingerlings during or after an outbreak but the prevalence of seropositive fish is very low and it is uncertain whether these are survivors of an outbreak of EHNV infection ([Whittington et al. 1994; 1999](#)) or are infected with another, less virulent ranavirus. Nevertheless, the high case fatality rate and low prevalence of EHNV infection in rainbow trout means that the recruitment rate of carriers is likely to be very low (< 2%). Further longitudinal studies will be required to confirm the poor potential for rainbow trout to act as carriers of EHNV.

Because of its extreme susceptibility it seems unlikely that the redfin perch could be the natural host or a suitable reservoir host for EHNV. There may be an as yet undiscovered natural host that is responsible for infection of redfin perch, which simply amplify the infection in an ecosystem. However there is some conflicting evidence. The EHNV or a related ranavirus was isolated from 2 of 40 apparently healthy adult redfin perch during epizootics in juveniles in Victoria ([Langdon and Humphrey 1987](#)) and several

ranavirus isolates have since been obtained from redfin perch in Victoria at times when there was no obvious epizootic. As the incubation period extends for up to 28 days (Whittington and Reddacliff 1995), there needs to be cautious interpretation of results from point-in-time observation (Langdon and Humphrey 1987). Some apparently healthy redfin in Victoria had serum antibodies against EHNv or a related virus (unpublished data). Neutralising antibodies against ranaviruses have not been detected, but it is conceivable that infection with an avirulent ranavirus could confer immunity against a virulent virus. Insufficient numbers of fish and ranavirus isolates have been examined to rule out the existence of ranavirus strains of low virulence in southeastern Australia and transmission trials in naive fish will be required to confirm the pathotype of isolates acquired from apparently healthy populations.

Effect of environment on disease expression

Natural epizootics of EHNv affecting juvenile and adult redfin perch occur mostly in summer (Langdon et al. 1986b; Langdon and Humphrey 1987; Whittington et al. 1994). This seasonal occurrence has not been explained, although it has been assumed that the disease in juvenile fish is related merely to the annual appearance of large numbers of non-immune young fish and their subsequent exposure to the virus while schooling in shallow waters; adults are uncommonly involved in these outbreaks. It is possible that environmental temperature is the trigger for EHNv outbreaks in redfin perch. Juvenile fish tend to feed in warm shallow waters on planktonic fauna whereas adult fish feed on benthic invertebrates and larger prey in deeper cooler water. Depending on the water body and the degree of thermal stratification with depth of water, these behavioural factors could result in exposure of different age classes of the population to different temperatures. Perhaps in this way adult fish may avoid the disease (Whittington and Reddacliff 1995). Temperature effects were evaluated experimentally in seronegative adult redfin perch collected from a healthy wild population and acclimated in our laboratory aquarium facility for 5 months at 18°C. In preliminary trials, fatal disease was invariably produced within 28 days at 12-19°C regardless of the route of inoculation. After bath exposure at water temperatures below 12°C, these fish did not succumb to EHNv but they were susceptible when exposed at temperatures $\geq 12^\circ\text{C}$. Fish exposed and held at 8-10°C were alive 16 weeks later, and were not affected within 7 weeks of re-exposure, but succumbed to a further exposure after the water temperature had been increased to 12°C. Fish exposed at 6-7°C were alive 5 weeks later and did not succumb

within 6.5 weeks of increasing the temperature to 19-21°C, but all died after re-exposure to EHNv at this higher temperature. The incubation period ranged from 10-28 days at 12-18°C compared with 10-11 days at 19-21°C (Whittington and Reddacliff 1995). Langdon (1989) reported an incubation period of 3-6 days after IP or bath exposure of juvenile redfin perch kept at 18-24°C. The survival of redfin perch exposed at water temperatures below 12°C was not associated with production of specific serum antibody although seroconversion occurs when redfin perch are injected with inactivated EHNv (Whittington and Reddacliff 1995).

In rainbow trout, water temperature also appears to influence the incubation period after IP inoculation; it was 3-10 days at 19-21°C compared with 14-32 days at 8-10°C (Whittington and Reddacliff 1995). However, outbreaks have been seen on farms over a range of temperatures from 11-20°C (Whittington et al. 1994; 1999). Natural outbreaks appear to be related to poor husbandry, particularly overcrowding, inadequate water flow and fouling of tanks with feed. Water quality parameters are suboptimal and intercurrent diseases, including skin diseases caused by protozoa and fungi and systemic bacterial infection are common. Damage to skin may provide a route of entry for EHNv. Mortalities tend to cease when stocking rates are reduced and water quality is improved.

Resistance of the virus

The EHNv is extremely resistant to drying and in water can survive for months. It can persist in frozen fish tissues for more than 2 years (Langdon 1989) and frozen fish carcasses for at least a year (Whittington et al. 1996). It is susceptible to 70% ethanol, 200 mg•L⁻¹ sodium hypochlorite or heating to 60°C for 15 min (Langdon 1989).

Transmission

The occurrence of EHNv in redfin perch in widely-separated river systems and impoundments and upstream progression indicates that the spread of virus cannot be due only to the movements of water (Fig. 2). The EHNv may be spread by migrations of fish in rivers. Redfin perch migrations in Australia are uncertain, but native species such as golden perch may travel more than 1000 km while Murray cod, a potential carrier species, travel at least 214 km (Reynolds 1983). Being a resistant virus, EHNv could also be moved from one place to another on nets, boats and other equipment. Recreational fishermen move live redfin perch between river systems and impoundments for sport despite recommendations to the contrary and catch, freeze and store redfin perch for use as bait elsewhere. Birds are potential

mechanical vectors for virus in the gut, on feathers, feet and the bill. Silver gulls (*Larus novaehollandiae*) and great cormorants (*Phalacrocorax carbo*) feed on affected juvenile redfin perch and the gastrointestinal contents of these birds were positive for EHNv in ELISA and PCR. The virus tends to become inactivated at the body temperature typical in birds (40 - 44.4°C), but spread of EHNv by regurgitation of ingested material within a few hours of feeding is possible (Whittington et al. 1996).

The means of spread of EHNv between rainbow trout farms is most likely due to transfer of virus with infected fingerlings and transport water. There is epidemiological evidence for this in the form of fish trading histories for several affected farms (Langdon et al. 1988; Whittington et al. 1994; 1999). The low prevalence of infection in rainbow trout means that active infection can easily go unrecognised in a population and be spread by trading such fish, especially since the numbers traded are usually specified in thousands.

There are no data on possible vertical transmission of EHNv on or within ova and disinfection protocols for ova have not been evaluated. The EHNv has not yet been isolated from ovarian tissues or from broodstock, therefore milt and ovarian fluids may not be suitable samples for testing for exclusion of EHNv.

Types and characteristics of diagnostic tests

Laboratory tests are required to confirm infection with EHNv and to conduct certification for movement of farmed fish. The tests include cell culture, immunological tests for antigen and antibody and DNA-based detection methods. General methods for international use are given in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000).

Cell culture

This is the gold-standard test but is costly and time consuming. The EHNv grows well in many fish cell lines at a range of temperatures but Bluegill Fry cells (BF-2, ATCC CCL 91) are preferred and an incubation temperature of 22°C both before and after inoculation with virus is important if results are to be repeatable (OIE 2000). The identity of viruses in cell culture is determined by immunofluorescence, ELISA, electron microscopy or other methods. A simple method for preparation of fish tissues for cell culture has been validated (Whittington and Steiner 1993).

Immunological tests

Polyclonal antisera were raised in rabbits and sheep and a range of test protocols were developed by the OIE Reference Laboratory. These include immunofluorescence and immunoperoxidase stains for histological sections and cultures, ELISA for use with cell cultures and homogenates of fish tissues and immunoelectron microscopy (Hyatt et al. 1991; Steiner et al. 1991; Hengstberger et al. 1993; Whittington and Steiner 1993). The analytical sensitivity of ELISA is 10^3 to 10^4 TCID₅₀•ml⁻¹. Specificity approaches 100% and sensitivity 60% (unpublished data). False negative ELISA results were obtained from fish that contained small numbers of infectious particles (Whittington and Steiner 1993). The ELISA is useful for both diagnosis and certification. Neutralisation tests cannot be used to identify EHNV because neutralising antibodies are not produced following immunisation of mammals or fish. Mouse monoclonal antibodies produced against EHNV are directed against MCP epitopes and are non-neutralising (unpublished data).

Serology

Anti-EHNV antibody can be detected in fish serum by ELISA using specific anti-redfin perch or anti-rainbow trout immunoglobulin reagents (Whittington et al. 1994; Whittington and Reddacliff 1995). Competitive ELISA has also been established (unpublished data).

Polymerase Chain Reaction (PCR) and other DNA tests

A PCR using primers for an unknown region of approximately 300 bp amplifies DNA from a broad range of iridoviruses including EHNV, BIV, ECV, ESV and FV3. It does not amplify lymphocystis virus or reptilian erythrocytic iridovirus (Gould et al. 1995). Primers specific for MCP can also be used to detect and rapidly differentiate the sub-groups of ranaviruses (Table 2; Fig. 3) (Marsh et al. 2002). The method requires extraction of DNA from a cell culture preparation, two PCR assays for regions of MCP and restriction endonuclease analysis using 4 enzymes. It can be completed in less than 24 hrs at relatively low cost. The European, North American and Australian viruses are clearly distinguished by this method. Furthermore, EHNV and BIV are readily differentiated (Table 2). The sensitivity of PCR in diagnostic applications directly on fish tissues is being evaluated.

Table 2. Rapid differentiation of ranaviruses. Each viral DNA extract is examined in two PCR reactions (PCR-1 and PCR-2) which target different parts of the major capsid protein gene, followed by restriction endonuclease analysis with the enzymes shown (Marsh et. al. 2002). The letters (A to M) given under each restriction enzyme correspond to the patterns shown in fig. 3

Patterns shown in Fig. 3							
Virus	Isolated from	Location	Restriction enzyme pattern				Profile
			PCR-1		PCR-2		
			<i>Pfl</i> MI I	<i>Hinc</i> II	<i>Acc</i> I	<i>Fnu</i> 4H I	
EHN ^V	Fish	Australia	A	C	F	I	1
BIV	Amphibian	Australia	A	D	G	J	2
FV3	Amphibian	America	B	D	H	K	3
GV	Amphibian	America	B	D	H	K	3
ESV	Fish	Europe	B	D	G	L	4
ECV	Fish	Europe	B	D	G	L	4
WV	Reptile	Irian	B	E	G	M	5
		Java					

Disease control and certification

Control in farmed fish

The EHN is endemic in southern Australia and cannot be eradicated from farmed fish because free-living species are involved. The disease causes few problems on infected farms and its significance lies with the implications for trade and the possible effects on native free-living species, some of which are vulnerable or endangered. Disease control at the farm level relies on reducing the impacts of infection by maintaining low stocking rates and adequate water quality. The mechanism of protection may be through maintenance of healthy integument.

Regional control

Animal disease control policy in Australia is coordinated nationally but determined at a state level. A draft policy exists in New South Wales to create an endemic infection zone in the southeast. This zone includes the catchments of all known infected fish farms and all impoundments where the disease EHN is known in redfin perch. Endemically infected farms are permitted to sell killed fish and fillets for the table. Unless health certification is undertaken these farms are not permitted to export live fish product from this zone but are free to distribute live fish or ova within the zone. Government-owned salmonid farms within the endemic infection zone in New South Wales undertake annual certification testing for EHN prior to distribution of fish or ova. This testing involves sampling sufficient fingerlings to detect 2% prevalence of infection with 95% confidence. Tasmania and Western Australia remain free of EHN by geographic isolation and controlling imports of live fish from other states. Western

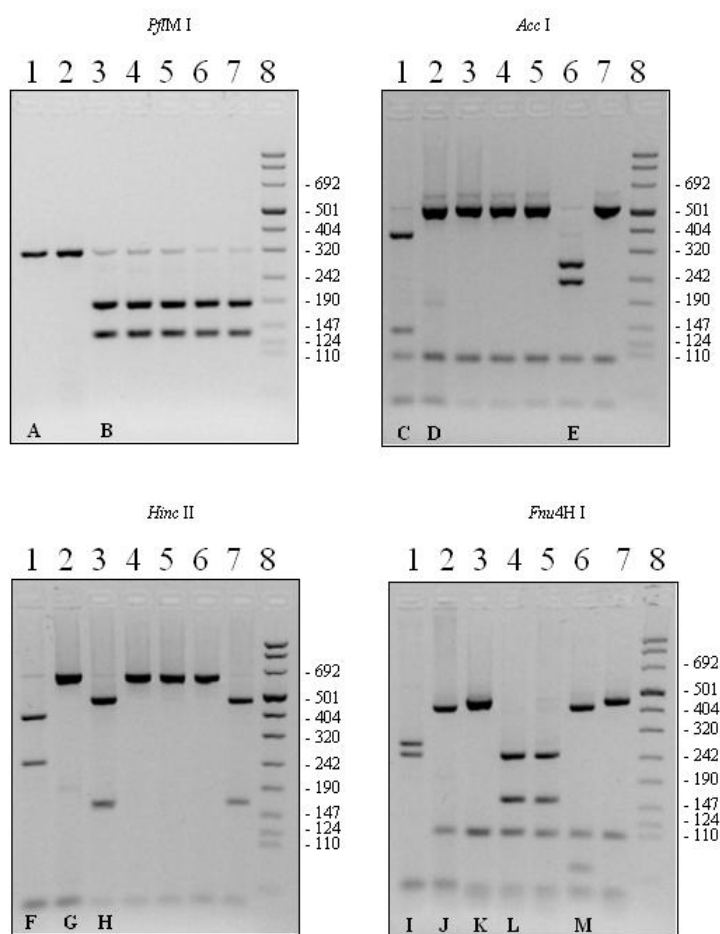


Figure 3. Gel electrophoresis of ranavirus major capsid protein PCR products after restriction with the enzymes shown. The samples are from lanes 1-7: EHNV, BIV, FV3, ESV, ECV, WV and GV. Lane 8 is the molecular size marker. The letters (A to M) refer to patterns that are used to identify the viruses as described in [table 2](#).

Australia has an active surveillance program for EHNV in salmonids, using ELISA while Tasmania has active surveillance using cell culture for listed viral diseases.

Emergency response

Disease incursions need to be detected rapidly using specific diagnostic tests. Screening by ELISA should be available to facilitate early diagnosis, with confirmation by cell culture. The ELISA developed for EHNV detects ESV, ECV and amphibian/reptile ranaviruses. Rapid confirmation of the subgroup of ranavirus can be achieved by PCR. This will

be important in regions where EHNv is absent, but ESV/ECV is present, for example Europe. The only possible means of eradication is by destocking, and this could be contemplated where the virus has been detected before likely spread to wild fish stocks. Stock should be burnt or limed and buried. The farm then requires thorough disinfection. Sodium hypochlorite ($200 \text{ mg} \cdot \text{L}^{-1}$) is effective in the absence of organic matter (Langdon 1989). Porous materials and timber should be burnt. Ponds should be drained and the water disinfected by chlorination prior to discharge. Ponds should then be cleaned and treated with hypochlorite. Pond sediments should be limed and buried. Equipment should be disinfected with hypochlorite after removal of organic matter. The movement of stock, personnel, equipment and water from the farm should be assessed and controlled. A quarantine area should be established to encompass nearby fish farms and surveillance of these should be established. Other farms, identified by trace-back and trace-forward of stock movements from the affected farm should be quarantined and monitored. The success of eradication can be assessed after restocking with disease-free fish. It may be necessary to undertake successive rounds of testing to prove this. If eradication is unsuccessful an endemic disease zone can be established to contain the infection and permit trade from disease-free farms outside the zone.

Restriction of spread of EHNv

Movement controls, certification testing and surveillance testing are required. Quarantine periods required to detect EHNv in carrier or subclinical stages of infection cannot be specified; the incubation period in the most susceptible species, redfin perch can extend to 28 days. Caution is required in purchase of fish from non-certified farms within the endemic infection zone. Farm history, clinical examination, the results of passive surveillance for disease agents and active surveillance for EHNv can be used to claim freedom from EHNv, however, active surveillance is required in most instances.

Surveillance and certification for EHNv

Statistically valid sampling practices need to be used and the correct organs/samples need to be collected. There is no evidence of reproductive tract infection and broodstocks are not known to participate in an infection cycle so milt and ovarian fluid are unsuitable samples. Standardised tests of specified sensitivity and specificity should be used. The chances of detecting EHNv infection in apparently healthy rainbow trout is extremely low, even where disease is active in the same population, because the prevalence of infection is low and there is a high case fatality

rate. For practical purposes, EHNv can only be detected in fish that are clinically affected or that have died with the infection. Thus routine certification practices based on the examination of large random samples of the entire population are not appropriate for EHNv. From a random sample of live fish it would be possible to misclassify a farm as being free of EHNv even during an outbreak of the disease because the prevalence of infection is generally very low. Examination of 'routine' mortalities is recommended. During an outbreak of disease, the prevalence of EHNv among mortalities may be 60-80% and the contribution of EHNv to 'background' mortality on an endemically infected farm is high enough to enable detection of the virus in the absence of overt disease in the population. For EHNv detection and certification purposes, the population of real interest is 'the population of mortalities' and sampling rates can be selected to detect at least one EHNv-infected individual at a given level of confidence given a certain prevalence of infection and test sensitivity (Cannon and Roe 1982; Simon and Schill 1984). For EHNv detection and certification in New South Wales, we assume a prevalence of 2%, based on data which suggested that at least 2% of 'routine' mortalities on an endemically infected farm were associated with EHNv infection (Whittington et al. 1999). The size of the population of 'routine' mortalities is difficult to measure as it increases daily, but if it is assumed to be very large, a conservative outcome with respect to confidence will be obtained. The antigen-capture ELISA used to screen tissue homogenates for EHNv has a sensitivity of at least 60% compared to cell culture (Whittington and Steiner 1993). The sample size required from a very large population of 'routine' mortalities to provide 95% confidence in detecting at least one infected individual using a test of 60% sensitivity is approximately 250. In practice, we specify that 'routine' mortalities be collected daily and stored in plastic bags in groups of 20 at -20°C until a sample of 250 has been gathered. Where possible, we target young age classes to simplify dissections and tissue processing. Individual clarified homogenates that are positive in antigen-capture ELISA are then subjected to cell culture to confirm the presence of EHNv. This is an economical approach as it greatly reduces the number of cell cultures required.

Serology might also play a useful role in surveys to identify infected trout populations. Assuming a 1% prevalence of seropositive grower fish on an endemically infected farm, a sample of 300 fish would be required to be 95% certain of detecting at least one infected individual (Cannon and Roe 1982). Further research is required to confirm the validity of this approach.

Origins of EHNV

There was no obvious clinical evidence of EHNV in rainbow trout in Australia prior to 1987. A survey of rainbow trout was conducted between 1981 and 1984 to detect viral infection at 5% prevalence with 95% confidence on five trout farms that are now considered to be within the endemic infection zone. Tissues appropriate for EHNV detection were examined using appropriate cell culture techniques but no viruses were isolated (Langdon et al. 1986a). Notwithstanding the difficulties of detecting EHNV in a survey of this kind, it appears that this infection was not present in trout at that time. Redfin perch populations were not surveyed until after the first outbreaks of EHNV infection (Whittington et al. 1996).

The origins of EHNV and related viruses of fish are uncertain. They appeared suddenly in developed countries in the 1980's associated with spectacular mortalities in fish which could not have been overlooked in the past. Arguably the redfin perch is too susceptible to be the natural host and rainbow trout too inefficient in virus propagation. It was assumed that trout became infected by exposure to redfin perch. There has been one unsuccessful attempt to find EHNV in a survey of invertebrate hosts (Langdon 1989).

There is lack of correlation between the commercial trading movements of food fish and recent iridoviral epizootics. Following isolation of ranavirus from aquarium fish, it was suggested that these viruses are being spread globally by the trade in ornamental fish (Hedrick and McDowell 1995). Ranaviruses have also been recovered from frogs, toads and other amphibians, but not necessarily associated with disease. The virus isolated from tadpoles of the ornate burrowing frog in Australia, BIV, caused systemic infection in several other species of frogs (Cullen et al. 1995; Cullen and Owens 2002) and also in barramundi (*Lates calcarifer*) (Moody and Owens 1994) and possibly tilapia (*Oreochromis mossambicus*) (Ariel and Owens 1997). In America ranavirus was isolated from dead or moribund stickleback (*Gasterostelus aculeatus*) and red-legged frog tadpole (*Rana aurora*) from the same habitat confirming that exchange of virus between amphibians and fish can occur in nature (Mao et al. 1999). Lack of host specificity, variation in virulence between hosts and the isolation of a ranavirus from a diseased python smuggled into Australia from Irian Jaya (Indonesia) (Hyatt et al. 2002) adds weight to the suggestion that trade or movement of amphibians and reptiles might explain the occurrence of ranavirus infections in fish in several developed countries. Whether amphibians and reptiles are the natural reservoir of these viruses is uncertain. There is a strong argu-

ment in favour of environmental surveys to screen populations of amphibians and reptiles for EHNV-like viruses.

Conclusion

The EHNV is an indiscriminate pathogen, lacking host specificity but displaying considerable variation in virulence in different species of finfish. Detection of EHNV in rainbow trout can be difficult because infection need not be associated with clinically detectable disease in the population. However, EHNV can be detected in a small proportion of 'routine' mortalities and may be associated with specific antibodies in a small proportion of older fish. Sampling to detect EHNV for certification purposes should be based on examination of 'routine' mortalities rather than random samples of live fish. Further studies are needed to identify the environmental reservoir of EHNV and other fish ranaviruses and to check for the presence of other ranaviruses within the EHNV endemic zone. This will enable more meaningful assessment of the role of legal and illegal trade in food fish, ornamental fish, reptiles and amphibians in the spread of these viruses. Government policy decisions related to detection and control of EHNV should follow a comprehensive survey of the distribution of EHNV in southeastern Australia.

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