Final Report

for

Diagnosis and Classification of Epizootic Haematopoietic Necrosis Virus (EHNV)

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This Project was commissioned by the Fishing Research and Development Corporation in 1989 and was completed in December 1992.

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Introduction

EHNV is an Iridovirus which has been isolated from redfin perch and rainbow trout. The first isolation was reported by Langdon *et al.* (1986) from redfin perch collected from Lake Nillahcootie during November and December,1984. The virus was identified by Dr A.D. Hyatt at AAHL.

The disease gained prominence because of the characteristic high mortalities of juvenile fish and necrosis of the renal haematopoietic tissues including the liver, spleen and kidney in previously unchallenged adults (Langdon and Humphrey, 1987). To date, outbreaks in redfin perch have occurred in isolated Victorian farm dams, Ovens River (Vic. December, 1985), Goulbourne River (Vic. 1986), Winneke Reservoir (Vic. 1988), Lake Nillahacootie (Vic, 1984,85), Lake Hume (Vic. 1984,85), Lake Makoan (1984,85,88), Blowering Reservoir (NSW, 1987), Mount Bold Reservoir (S.A., 1991), Lake Alexandrina (S.A., 1991) and Mount Albert Reservoir (S.A., 1991) Reports have indicated that the virus is now spreading upstream (as far as Renmark) from the lower reaches of the Murray river. Prior to the more recent outbreaks of the disease, it was believed that EHNV was restricted to waters of the upper Murray. As has been the case in other outbreaks the disease in S.A. has resulted in the near decimation (95%) of redfin populations over short periods of time (three weeks). Outbreaks in redfin have been reported in the south west slopes, southern ranges and western plains regions of NSW, the virus is believed to be spreading progressively in NSW waterways. Outbreaks of EHNV have also occurred in rainbow trout farms in NSW (1986,87,90) and in Vic. 1988. Disease similar to that of EHN has been observed in diagnostic samples from trout farms in Vic. during 1990,91.

The virus has also been transmitted (under laboratory conditions) to a range of native and introduced teleosts including Macquarie perch (*M. australasica*), silver perch (*Bidyanus bidyanus*), mosquito fish (*Gambusia affinis*), mountain galaxias (*Galaxias olidus*) and Murray cod (*Macullochella peeli*), (Langdon *et al*, 1988). The susceptibility of native fish to EHNV may explain the decline in population numbers of various species over the last decade. The disease posses serious consequences for the conservation of our native teleosts, the movement of fish throughout Australia and the export industry in eyed trout ova from south-eastern Australia to New Guinea, Europe and the Middle East.

The threat of the disease at both the national and international levels has been recognised by the OFFICE INTERNATIONAL DES EPIZOOTICS in the classification of the disease as a list B disease. Such classification places stringent conditions on the classification of EHNV free countries, zones and farms. That is, the movement (importation) of live fish of any species, or their spawning products (eggs, ova, milt) must be accompanied by an international animal health certificate from the exporting country certifying that the consignment originates from either a country declared to be **EHNV-free** or from a farm officially declared **EHNV-free**. The issue of such certificates will arise from nationally accepted standardised diagnostic tests and statistically valid sampling regimes.

At the onset of this project it was apparent that there was a requirement for the development of a range of diagnostic tests whereby EHNV and exotic viruses could be detected. To achieve these objectives it was necessary to perform some basic research into the replication of EHNV at the cellular level. This informationwas vital for the eventual production of highly purified viruses and thereby high titered antibodies. The results obtained during the course of this project are listed and discussed below. Collectively, they showed (a) a good understanding of the structure and replication strategy of EHNV at the cellular level; (b) EHNVto be a iridovirus belonging to the genus *Ranavirus* within the family *Iridoviridae*; (c) antibodies which were produced against two isolates of EHNV (rainbow trout and redfin) were high titered, clean, specific and sensitive; (d) that we now have a battery of robust diagnostic tests for the detection of EHNV DNA and virions in cells, supernatants and clinical samples in addition to antibodies from the serum of EHNV-exposed fish; (e) a high degree of similarity between EHNV and iridoviruses isolated from a native Australian frog; (f) a wide distribution of EHNV

in redfin throughout the waterways of Victoria and (g) the production of sensitive electron microscopical and ELISA tests for the detection of IPN.

Objectives

The objectives which were originally defined in the project are listed below.

1. To examine the biology of EHNV.
2. To apply the knowledge to the development of rapid diagnostic tests, namely immunoelectron microscopy (IEM) and the polymerase chain reaction (PCR), which in collaboration with the Elizabeth McCarthur Agriculture Institute an antigen-based enzyme-linked immunosorbant assay (ELISA) which collectively will provide a comprehensive battery of diagnostic tests for EHNV.
3. Use the technologies developed in objectives 1&2 for the rapid detection of exotic fish viruses.

Staff

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- Mr S. Vallis; Arthur Rylah Institute, Heidelberg, Victoria.
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- Mr R. Whittington; Elizabeth Macarthur Agricultural Institute, Cambden, N.S.W. (EMAI)

Results

Results from the project are outlined below. For more details on any specific aspect of the work refer to the scientific papers which we have published during the course of this project.

Cell biology of EHNV

Cell susceptibility Two isolates of EHNV, one from rainbow trout, hereafter referred to as $EHNV_T$, and the other from redfin perch, hereafter referred to as $EHNV_{RF}$, were inoculated onto a range of mammalian, piscine and insect cells. The isolates replicated in all but the insect cell line. There was no differences between the isolates. These results are discussed later in comparisons of EHNV with other iridoviruses.

Structure of EHNV Ultrastructurally, $EHNV_{RF}$, $EHNV_T$ were very similar. Complete virions possessed an outer envelope (Fig. 1, 6) which was derived from the cell (plasma) membrane of the host cell. This outer envelope encompassed an inner icosahedral capsid, which is comprised of capsid subunits (refer below) containing a 51 kD protein (refer below) and a lipid membrane which in turn surrounds an inner core of double stranded DNA and associated proteins..

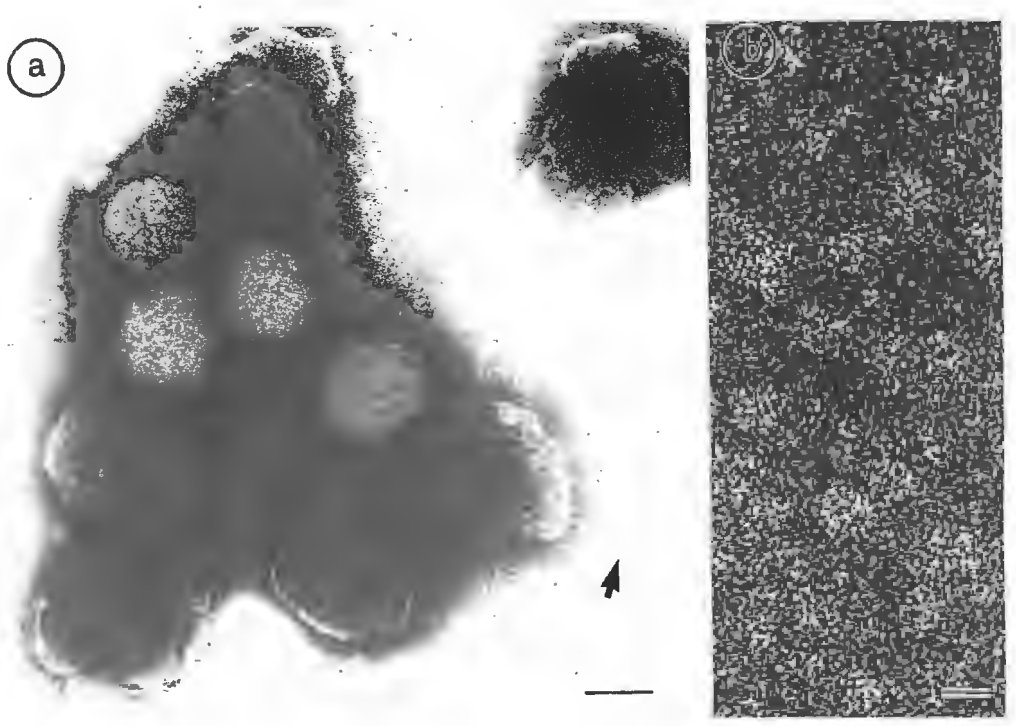

Fig.1 Transmission electron micrographs of negatively stained (a) purified EHNV and (b) capsid subunits: arrows, capsid subunits; bars in (a) and (b) represent 100nm and 20nm respectively.

When infected cells were extracted with a non-ionic detergent, viruses were observed to be associated with the filamentous framework of the cytoskeleton. Grid-cell-cultures of infected cells showed that EHNV isolates budded from the plasma membrane (Fig. 2), particularly along the long cellular processes of infected cells.

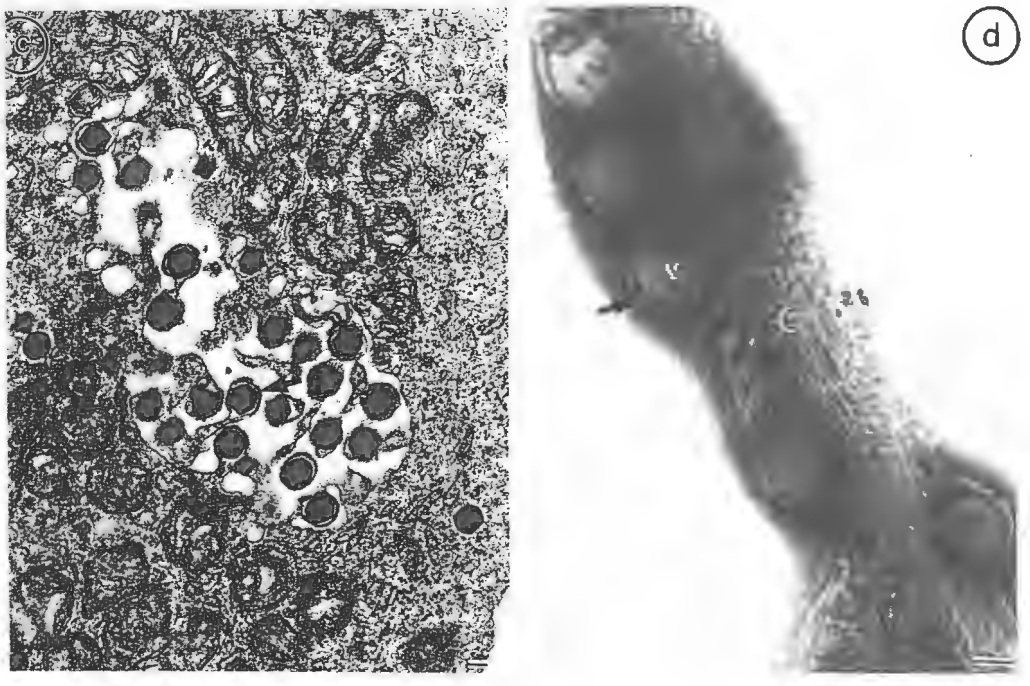

Fig. 2 Electron micrograph of EHNV budding from infected cells. (C) ultrathin sections of budding EHNV (arrow); (d) negatively stained specimen of budding EHNV; v, virus; C, cell. All bars = 100nm.

The location of EHNV within infected cells was investigated in terms of whether the virions were located in the soluble or insoluble fraction of host cells. Growth curve studies showed that a major proportion of infectious EHNV remained cellular associated and must therefore be physically sheared from the cytoskeletal framework of the cells if high numbers of viruses were to be purified. Results also indicated that to produce preparations of EHNV free from cell-associated debris then they should be subjected to treatment with a mild detergent; if this step was omitted then antibodies would be produced which would react with cell-associated antigens.

Purified virus preparations (Fig. 1) were also subjected to more intense treatments with detergent as to partially break down the viruses. The resultant preparation was sedimented through a sucrose gradient. This material was identified by electron microscopy as 7 to 9 nm capsid subunits and by polyacrylamide gels as the major 51kD protein of EHNV; these preparations (purified viruses and capsid subunits were used to generate antibodies (refer below).

Protein profiles of EHNV-infected cells and purified EHNV

The polypeptide composition of EHNV determined by polyacrylamide gel electrophoreses is shown in Fig.3. The protein profile of a Coomassie blue-stained SDS gel shows the presence of at least 19-20 polypeptides in purified viruses . The protein molecular weights (M.W.) range from 18Kd to 128Kd with the major proteins having M.W of 32Kd, 52Kd and 88Kd. Purified virus treated with NP40 (refer above) produced 7 to 9nm capsid subunits which when analysed by polyacrylamide gel electrophoresis were found to be comprised of a 52Kd proteins. Fig.3 also shows the [^{35}S]-methionine-labelled proteins synthesised in EHNV infected cells over a 16h period. The figure shows that host cell synthesis is shut down shortly after infection and that individual viral proteins appear at different times post infection. For example, a number of proteins first appear at 2 to 4 h p.i. (lane 2), their synthesis reaches a maximum at approximately 4 to 10h p.i. and they are not synthesised at all, or they are made at reduced levels, at times after 14h p.i. (lane 7). Proteins with M.Ws 25Kd, 31Kd, 31.8Kd, 33Kd, 41Kd, 44KD and 62kd fall into this catagory. Other viral proteins appear at approximately 4 to 6h p.i. (lane 3) and their synthesis is maintained throughout the infection, for example the 24Kd and 63Kd proteins. Finally other proteins do not appear in detectable amounts until 6 to 9h p.i. (lane 4), for example the 18Kd, 37Kd, 52Kd, 77Kd and 113Kd proteins. Such an ordered appearence of virus-specific proteins at different times p.i. is characteristic of many viruses including iridoviruses. Not surprisingly most proteins whose synthesis is maintained late in infection are incorporated into the virus, for example the 18Kd, 22Kd, 33Kd, 37Kd, 42Kd, 44Kd, 46Kd, 52Kd, 62Kd, 77Kd, 100Kd and 128Kd proteins. Several [^{35}S]-methionine-labelled intracellular viral proteins were not detected in purified viruses, for example 54Kd, 63Kd and 96Kd proteins; these proteins may represent non-structural proteins which are not incorporated into the virus particles. Conversely, some proteins were found in large amounts in purified virus (for example 32Kd and 88Kd species) but were either not found or were present in very small quantities (32Kd) among the intracellular viral proteins; this may simply reflect the amount of methionine in these proteins.

The polypeptide compositions of EHNV$_T$ and EHNV$_{RF}$ were also compared. Overall, there were only a few apparent differences between the profiles of the two piscine isolates (Fig 9). For EHNV$_T$ there were additional 37kD and possible 82kD proteins. However, these differences may be a reflection of the low amounts of proteins present and thus the inability to detect them may have been a quantitative problem. EHNV$_{RF}$ however, differed from EHNV$_T$ in the presence of an additional 31kD and 88kD proteins and the presence of a 72kD compared to a 73 kD in EHNV$_T$.

Nucleic acid (DNA) profile

Restriction enzymes *HpaII* and *MspI* were used to investigate the nature of the nucleic acid of EHNV. Iridoviruses from frogs, namely frog virus 3 (FV3, member of the genus *Ranavirus*) contain methylated DNA which possess the sequence CCGG which in turn is recognised by these enzymes. *Hpa*II will not however digest DNA if the internal cytosine (C) is methylated whereas *Msp*1 will. Fig 10 illustrates the susceptibility of EHNV DNA to digestion by *Msp*I and its resistance to *Hpa*II . Thus every internal cytosine residue in the sequence CCGG within EHNV DNA is

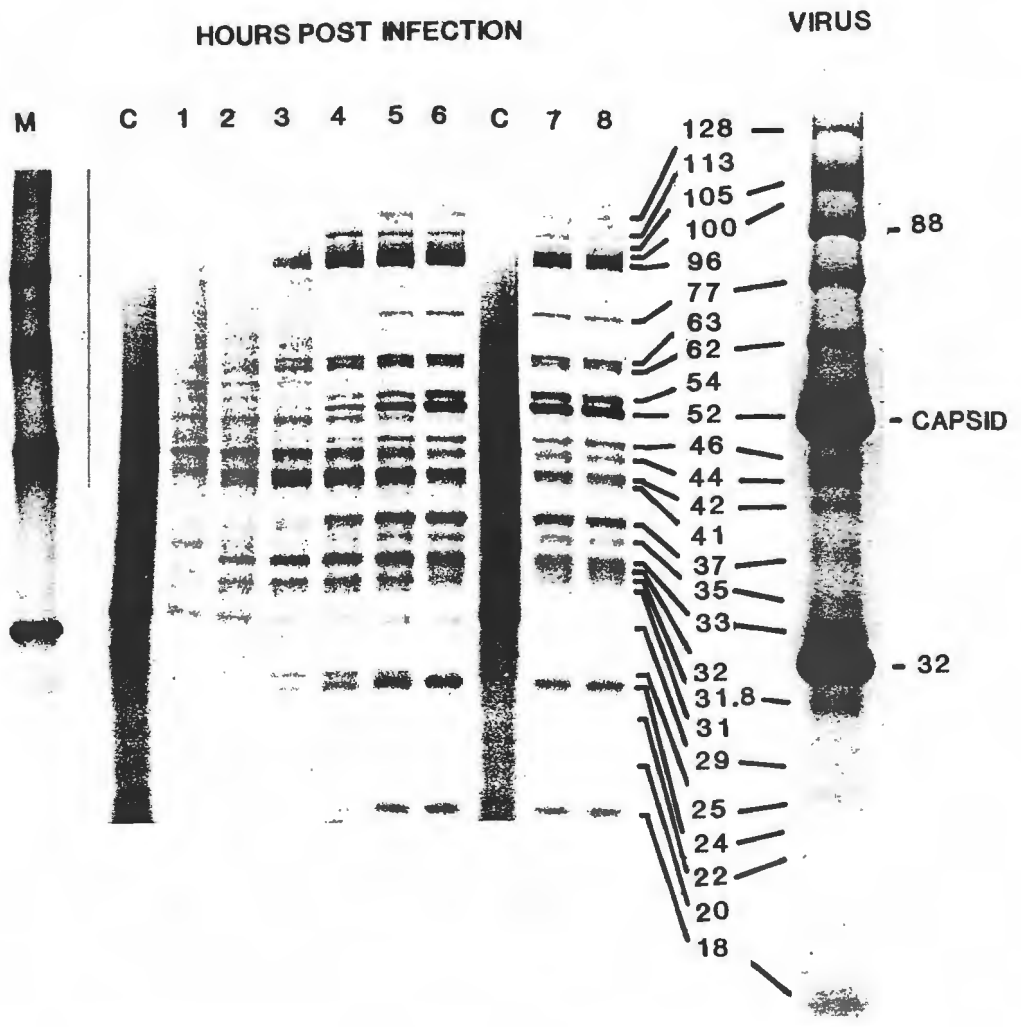

Fig 3. Proteins in purified EHNV and in EHNV-infected cells. Monolayers of BF2 cells were infected with virus and labelled with [^{35}S]-methionine at 2 h intervals for 16 h (lanes 1-8). Radioactively labelled proteins in uninfected (lane C) and infected cells and marker proteins (lane M) were detected by autoradiography. The estimated molecular weights of viral proteins are given in kD x 10^3. The proteins in purified EHNV were stained with Coomassie blue and their molecular weights estimated by reference to stained marker proteins run in the same gel.

methylated. These results showed that the genome of EHNV was similar to other described iridoviruses and had a size of approximately 125 kb. Furthermore the results indicate that EHNV is not similar to other described piscine iridovirses but resembles the amphibian virus FV3 (refer to section "Non-specified objectives" for further results).

Ultrastructure of infected cells

The ultrastructure of both $EHNV_T$ and $EHNV_{RP}$-infected cells were similar (Fig 4). Within the cytoplasm non-membrane bound viral inclusion bodies (VIB) appeared as rarified areas. These electron translucent structures were devoid of cellular organelles. Another prominent feature of the cytoplasm was the aggregation of mitochondria around the perimeter of the VIB. Within the VIB, areas of condensation, presumably DNA (Murti *et al*, 1985), were frequently observed. Within these structures large numbers of virions could be located, many of which were at different stages of developement, for example, capsid fragments , empty capsids and mature viral particles. Virus particles were also located singularly or in crystalline arrays within the cytoplasm. When cytoskeletons were prepared, viruses could be observed within the cells associated with 7-11nm filaments which constituted a sub-population of the overall cytoskeletal matrix of the cell, these filaments were also observed at the periphery of the inclusion bodies.

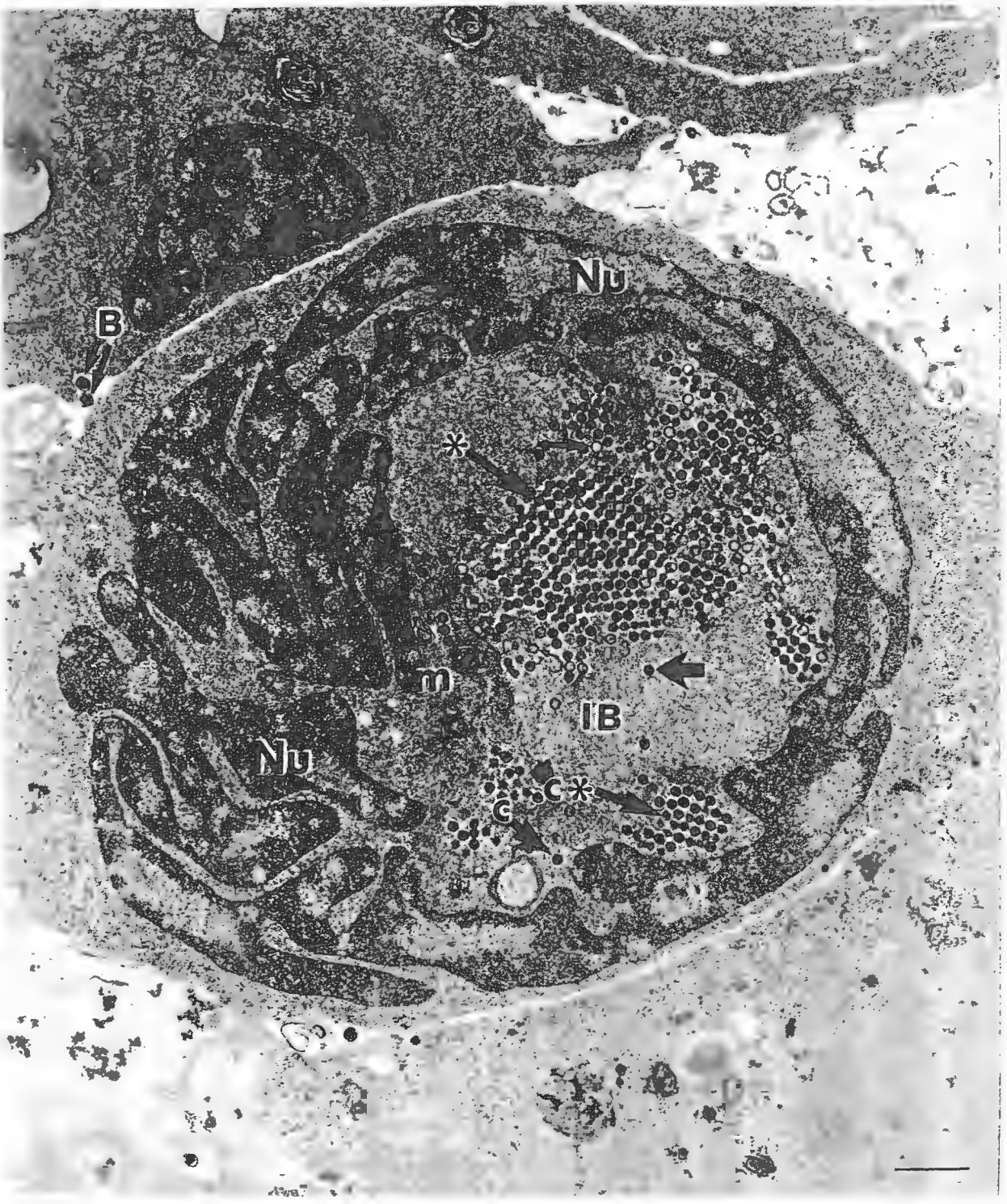

Fig.4 Transmission electron micrograph of an EHNV-infected cell. The various forms of developing virions are indicated by progressively-sized arrows: IB, inclusion bodies; m, mitochondria (bar 1μm).

At 20 h.p.i. virions were observed leaving infected cells and entering neighbouring uninfected cells. Viruses entered the cells by endocytosis and appeared to collect in lysozome-like bodies where the viruses appeared to undergo partial degradation. In the later stages of morphogenesis progeny viruses were released from the infected cells by budding. Whilst many viruses were observed budding from infected cells and acquiring an outer membrane, other viruses remained intracellular in the form of crystalline arrays or as individual cytoplasmic particles.

Development of rapid diagnostic tests for EHNV

The results described above provided a sound scientific background for the development of a range of diagnostic tests for the detection of EHNV. This basic research enabled (a) high numbers of purified EHNV to be generated free from adhering and contaminating cell-associated debris and (b) the subsequent generation of several clean and EHNV-sensitive antibodies. These antibodies were used in the development of antigen-capture ELISA's, immunoelectron microscopical and immunohistochemical diagnostic tests.

Antibodies: Examination by western blot analyses

Proteins from the $EHNV_{RF\&T}$ reacted with the antibodies generated from the whole virus and the purified 51kD subunits (Fig. 5). Western blot analyses showed that the antibodies from whole viruses reacted with a wide range of EHNV virus-specific proteins whereas antibodies raised to the 51kD protein recognised the 49-50kD, 44kD and 38kD proteins. Differences between $EHNV_T$ and $EHNV_{RF}$ were highlighted when denatured proteins from these viruses were reacted with antibody raised against the $EHNV_{RF}$. For example the 38kD protein from $EHNV_T$ reacted poorly with this antiserum whereas the homologous protein from $EHNV_{RF}$ generated an intense band and visa versa for the 44-45 kD band. Whether this difference is indicative of differences between the two isolates is at present unknown as many more isolates must be examined to confirm these findings.

Immunoelectron Microscopy

Purified $EHNV_{RF}$, $EHNV_{TF}$ were reacted with the various antisera and gold-labelled. Representative results are shown in Fig. 6. The gold-labelling was specific and clean. The pictures shown in Fig. 6 were derived from negative stained whole viruses. Archival resin embedded blocks were cut and incubated with the antiserum. Again good, specific gold-labelling occurred despite the use of concentrated fixatives and prolonged embedding times which are mandatory for conventional tissue processing. These results show the broad applicability and robustness of the antisera. As all antisera reacted equally well at the electron microscopical level, they could not be used to discriminate between the isolates.

Immunohistochemistry

Archival paraffin blocks of juvenile redfin perch were retrieved from storage for testing. These samples were part of a consignment submitted to the Australian Fish Health Laboratory (AFHRL) for EHN diagnosis. Incubation of sections from these blocks with the various antisera and florescence tagged secondary antibodies showed strong florescence within the haematopoietic tissues and the circulating red and white cells within the gills (Fig. 7). Analogous incubations with uninfected redfin and rainbow trout tissues were negative. These results show that the antibodies give specific results within clinical tissues.

ELISA Results

Whilst this project was operational, AFHRL was relocated to Geelong. Resultantly samples began to arrive over the summer months for EHNV exclusion and confirmation. The availability of highly purified EHNV (antigen) and high quality antisera were therefore used to develop an antigen-based capture ELISA. It was envisaged that this test would eventually be evaluated against a similar test being developed at the EMAI and the best of both tests combined (refer to section "Significance of Results"). The ELISA was tested at various levels. Table 1 shows the results of the ELISA for samples derived from tissue culture and the field samples where the fish displayed clinical symptoms of EHN. The test was also evaluated against both uninfected redfin and rainbow trout tissues. The test was further evaluated by analysing tissue samples from field survey work (refer below). The results were compared to those derived from classical virus isolation studies and PCR (refer below). The results showed that the ELISA was, in the main, more

reliable in the detection of EHNV than classical virus isolation procedures but not as sensitive as PCR. An explanation for the ELISA results may be the ability of the antigen-capture ELISA to detect infectious and non-infectious virions, soluble virus-specific proteins and degraded virus particles; the isolation of EHNV however depends on the presence of infectious virus particles. The EHNV antigen-capture ELISA is now in use by AFHRL at AAHL.

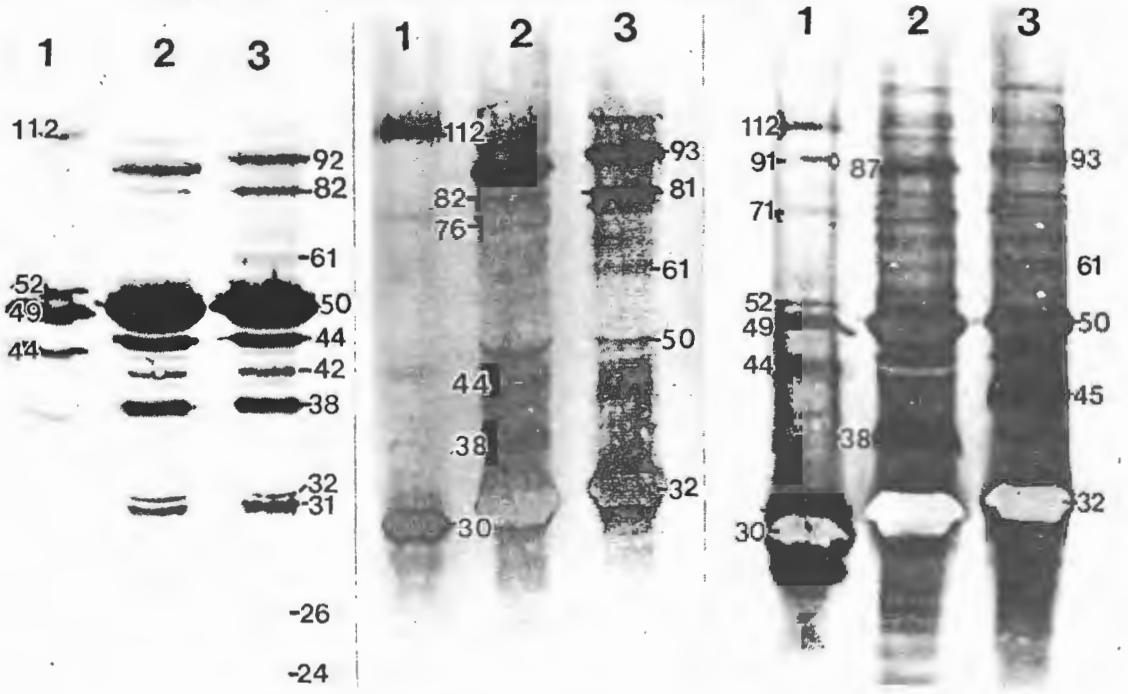

Fig. 5. Example of a Western blot analyses of EHNV proteins reacted with antisera raised against the 51-52kD capsid protein of $EHNV_T$ (A) and whole $EHNV_T$ (B) and $EHNV_{RF}$(C).

Polymerase chain reaction (PCR)

DNA was extracted from gradient purified EHNV by phenol/chloroform/SDS extraction followed by ethanol precipitation. DNA fragments generated by restriction enzyme digestion proved to be intractable to the methods generally used for cloning such fragments, presumably due to the high levels of DNA methylation. Therefore DNA was transcribed into complementary DNA using random hexamer primers and made double stranded by hairpin extension. The DNA was then digested with Sau3A and fractionated by passage through a 1% agarose-TAE gel. Selected bands in the 400 bp region were excised from the gel and purified using absorption to glass-milk. The eluted DNA fragments were then ligated into BamH1 digested pUC18 and inserts sequenced directly. The DNA sequences thus generated were analysed for open reading frames. Synthetic oligonucleotides were then synthesised using a DNA synthesiser, which were then used in PCR reactions to amplify the targeted DNA sequences. Several potential systems were trialled, however the least ambiguous signal was obtained using primers pAG133 and pAG134. These oligonucleotides specifically amplified EHNV from both redfin and trout, while no PCR product was seen with either the closely related Bohle virus or Lymphocystus virus (another iridovirus). PCR reactions were performed on clinical samples from fresh and frozen fish tissues. A summary of the results can be found in Table 2.).

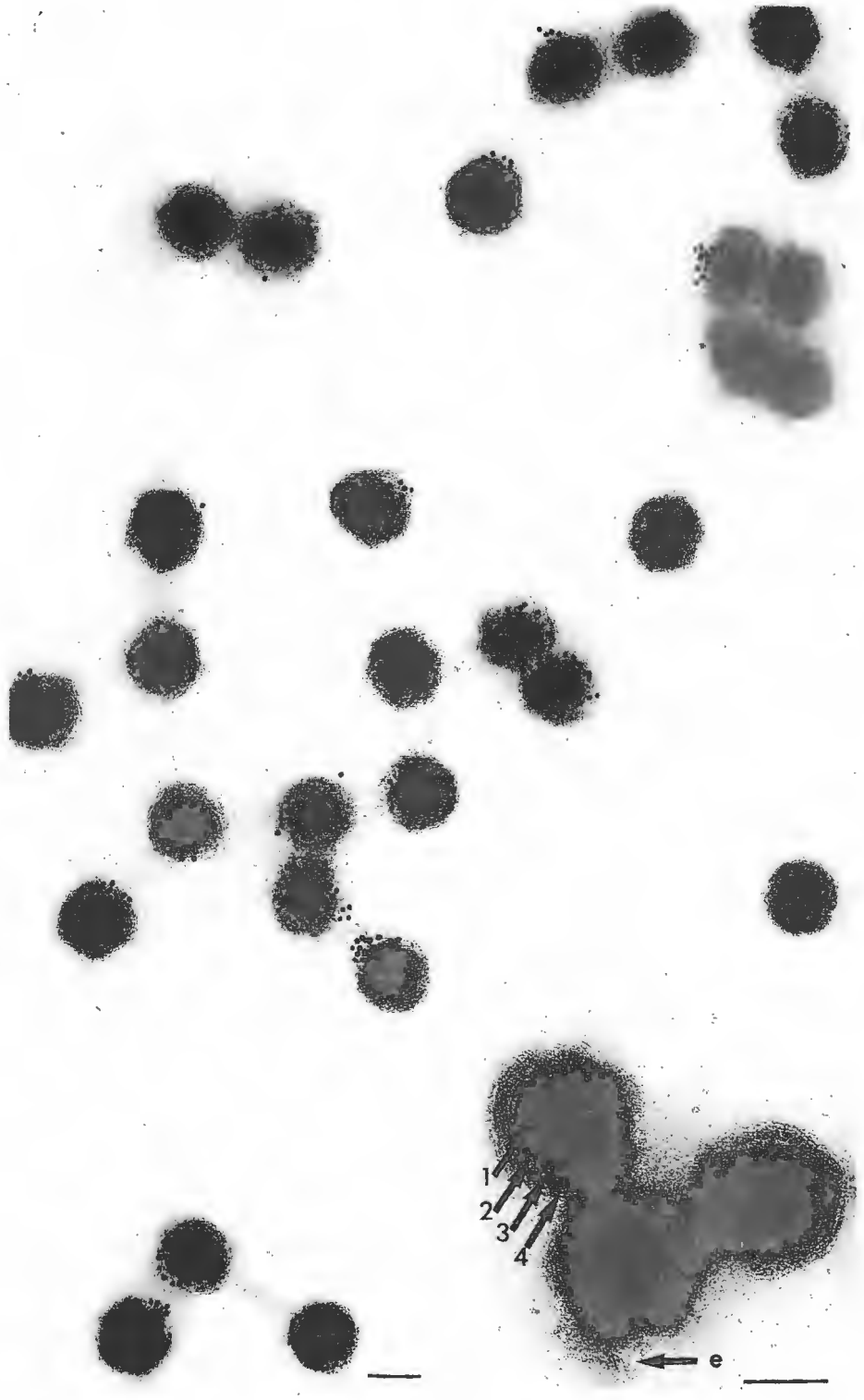

Fig. 6. Electron micrographs of gold-labelled EHNV(T). The insert is a higher magnification illustrating gold-labelling and the ultrastructure of the virus; (1) central core; (2) intermediate lipid membrane; (3) outer icosahedral shell consisting of 51kD proteins and (4) outer membrane derived from the plasma membrane. All bars = 100nm.

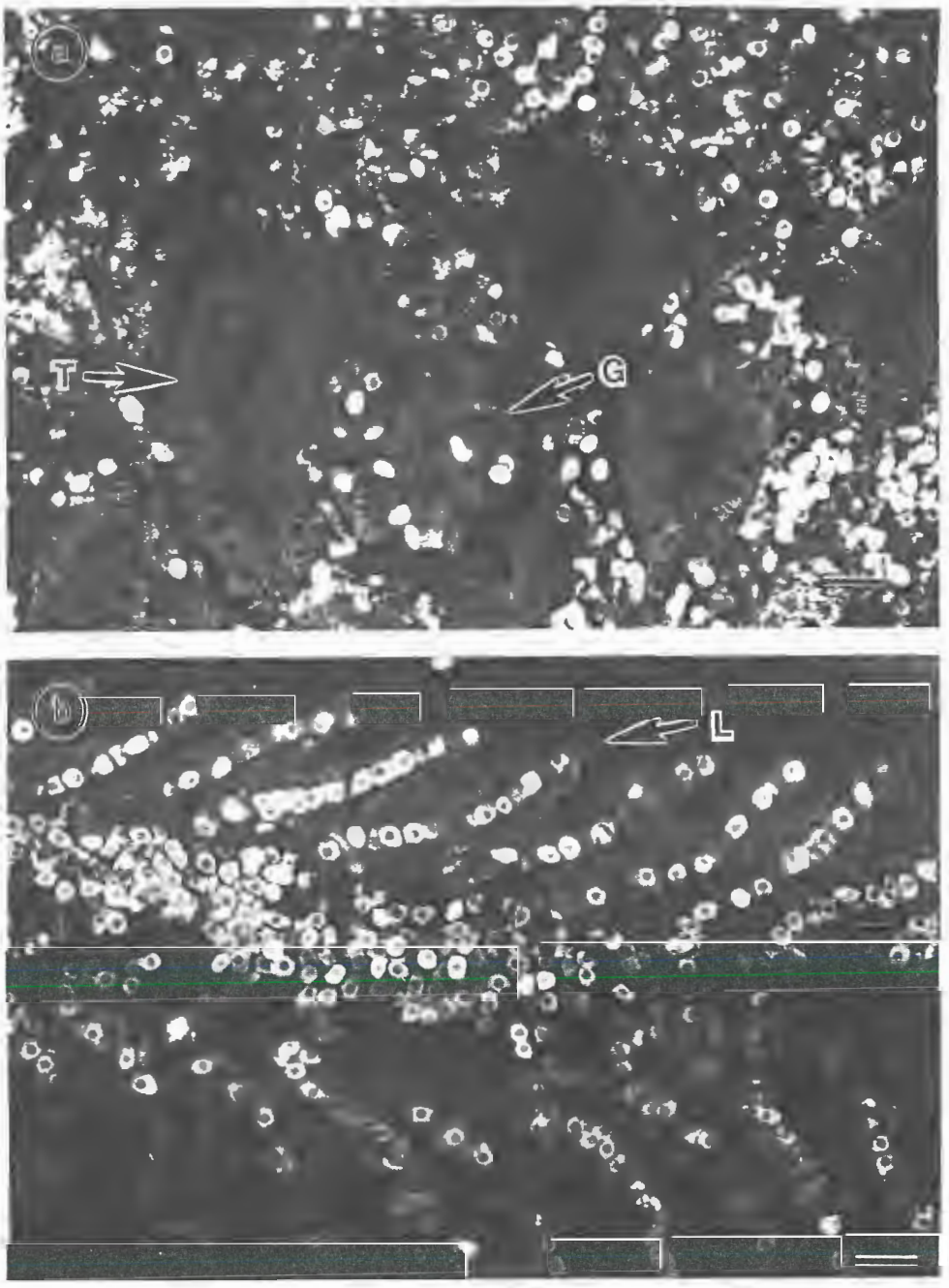

Fig.7 Sections of EHNV-infected juvenile redfin perch. (a) Section of kidney; T kidney tubules; G glomerulus. (b) Section of gill; l lamellae. Flouresence can be seen throughout the blood cells in both sections. Bars = 50 μm.

Table 1. Experimental results from antigen-capture ELISA

Sample	Disease state	Tissue	Dilution	O.D.(X±SE)	n
BF2 cells	Uninfected	T.C. super^{nt}	Neat	0.028±0.001	113
BF2 cells	Infected ($EHNV_{RF}$)	T.C. super^{nt}	Neat	1.484±0.065	55
			1:100	0.341±0.036	36
			1:150	0.151±0.031	14
			1:200	0.103±0.010	11
BF2	Infected ($EHNV_T$)	T.C. super^{nt}	Neat	1.052±0.037	32
			1:100	1.132±0.039	24
			1:150	0.053±0.023	10
Rainbow trout	Uninfected	Kidney, liver and spleen	1:10	0.036±0.000	16
			1:10	0.042±0.001	16
Redfin perch	Uninfected (T)	Kidney	1:10	0.002±0.000	36
			1:10	0.000±0.000	32
Redfin perch	Infected (H)	Kidney, liver and spleen	1:10	1.461±0.030	4
			1:80	1.287±0.031	4
			1.330	0.700±0.030	4

O.D. readings ar means (X) ± standard errors (SE) and n = total sample number. (T) indicates fish which were collected from Tasmania and (H) fish from an EHNV epidemic at Lake Hume. T.C. super nt represents tissue culture supernatant from infected cell monolayers.

Results from non-specified objectives involving EHNV

(i) Field related studies

During the course of the project diagnostic samples were submitted to this laboratory for EHNV exclusion and confirmation. These samples were processed by classical virus isolation procedures, convential electron microscopy and for testing with the newly developed antigen capture ELISA.

As the research proceeded contacts were made with the Arthur Rylah Institute and Snobs Creek. Scientists at these institutes offered to collect (a) at random and (b) in a systematic manner, redfin perch whilst conducting their own routine field trips. The sampling provided an indication to the distribution of EHNV throughout the waterways of Victoria. In the systematic studies conducted by Mr Peter Grant a methodical sampling survey of two known EHNV infected and one uninfected waterway(s) was performed. Details of the fish were collected as were the prevailing environmental conditions. Tissues (brain, gonads, intestine, pancreas, liver and kidney) were dissected from the fish and analysed by ELISA, and virus isolation. Various samples were chosen at random in addition to those which gave positive and/or equivocal results for analyses by PCR (Table 2).

The results summarised in Table 2 show that EHNV could be isolated from apparently normal fish and from a range of tissues. The data also showed the ELISA to generate positive results from tissues where viruses could not be isolated by classical means; PCR analyses of these same tissues confirmed the ELISA results. Data from PCR analyses illustrated that this is indeed a superbly sensitive test as it could detect EHNV DNA in samples which were negative by both virus isolation procedures and antigen capture ELISA assays. Of the tissues anaylsed from clinical cases, liver, spleen and kidney (anterior and posterior) appeared to be the better tissue for the detection of virus (Fig. 8). The gonads, on the otherhand, are not suitable for the detection of EHNV by either ELISA or PCR as they can generate spurious results.

EHNV DETECTION WITHIN MAJOR TISSUES

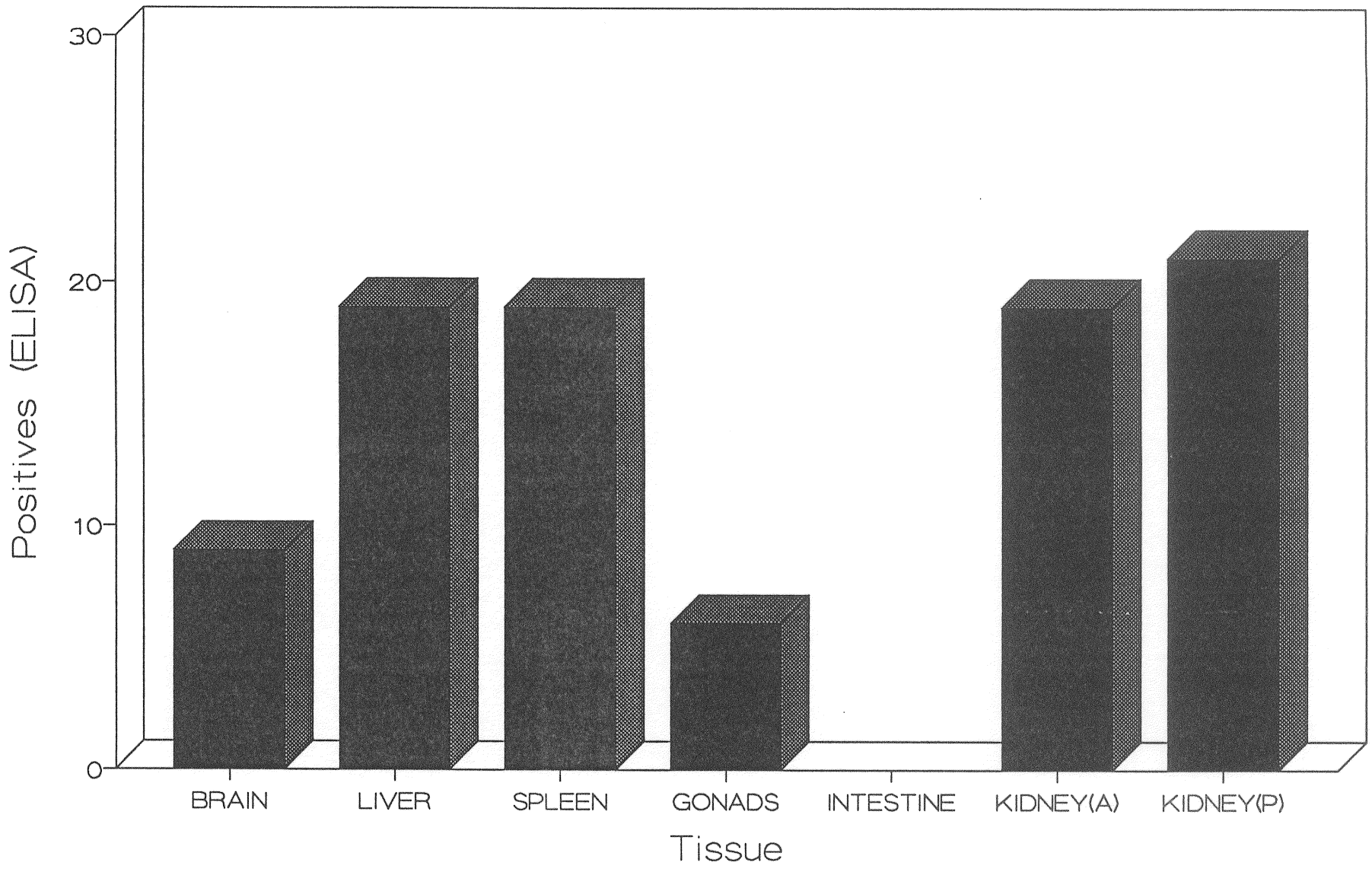

Fig 8

(ii) Distribution of EHNV throughout Victoria

The submission of samples by Snobs Creek and the Arthur Rylah Institute showed that EHNV is widely distributed throughout the various water basins of Victoria (including basins #7S, South Loddon; #4, Broken; #5 Golburne; #1W, Mitta Mitta; #36, Hopkins; #31, Werribee). It should be noted that during the course of this study, water-systems representing only a few water basins were sampled and that the majority of fish submitted for examination were redfin perch. These fish were in the main normal, that is, no clinical signs of EHNV were observed. The age of these fish were also estimated, they ranged from one to over five years in age. As many of these fish came from watersystems where epizootics have occurred (e.g. Lakes Makoan and Nillacootie) they may well represent survivors of these EHNV epizootics This statement is based on the age of the fish, location and time of last epizootic. Late in the course of this project the antigen capture ELISA was modified to an antibody-competitive ELISA whereby sera from any fish could be screened for the presence of antibodies against EHNV. This additional test was developed because we had received sera collected from live and released redfin perch (kindly supplied by Mr Peter Grant). Analyses of these sera showed the presence of antibody to EHNV; these results were, in the main, confirmed by Dr Richard Whittington at EMAI. Collectively, these results illustrated the applicability of the diverse range of diagnostic tests which we have developed at AAHL for the detection of EHNV within the tissues of field fish. The results also confirm that redfin perch can (a) survive EHNV infection (possession of antiserum against EHNV) and/or be carriers of EHNV. The presence of EHNV in healthy fish can also be interpreted to suggest that there are various serotypes of EHNV circulating in the field of which only a few are virulent. It should also be noted that although EHNV positive sera were detected in summer, autumn and winter, clinical EHN disease was only ever confirmed during the summer months.

During the course of systematic sampling (Lakes Makoan, Nillacootie, and Goulborne Laggon) environmental data or more specifically water quality data (water temperature, oxygen levels, conductivity, ammonia, nitrates, nitrites, pH and alkalinity) were collected. It was expected that over the period of two "EHNV seasons" (i.e. the summer periods) an outbreak would occur which would then permit contributing environmental conditions to be identified. Unfortunately this scenario did not occur and so critical changes in environmental conditions which contribute to the epizootics remain unidentified.

(iii) Comparisons of EHNV with an iridovirus (BIV) isolated from a native frog

During 1990 Dr Rick Speare from James Cook University, Townsville, isolated a virus from the ornate burrowing frog *Limnodynastes ornatus* (Gray). Dr Speare was informed of the work being performed at AAHL on fish iridoviruses and approached us to characterise this new amphibian virus called Bohle iridovirus (BIV). Described below are the results obtained from this study. The results are included in this report as they may have profound effects on the future distribution and virulence of EHNV viruses (refer below).

Ultrastructually, EHNV and BIV were very similar. Both isolates infected a similar range of host cells and replicated in a similar manner. However, when ultrathin sections were examined the size of BIV was found to be statistically smaller than that of EHNV (Table 4).

As was observed with cells infected with EHNV, BIV induced a rapid inhibition of host cell protein synthesis. Intracellular BIV proteins were determined by polypeptide gel electrophoresis and are shown in Fig. 9. The autoradiograph shows the presence of at least 20 polypeptides with molecular weights (MW) estimated to range from 22 to 123 kD. The number and size of BIV-specific proteins resembled that described for EHNV and FV3-infected cells (Eaton et al., 1991; Willis et al., 1977; Elliot and Kelly, 1980).

Table 2. Summary of survey work where the EHNV antigen-capture ELISA was assessed on clinical and apparent normal fish.

A. SURVEY																					
SAN	Location	Animals	Male	Female	Age 0-1	Age 2-3	Age 3-5	Age 5+	Number of tissues	+ve virus	+ve ELISA	+ve path	Age, sex (ELISA)						+ve PCR	Date	Species
													0-1	1-3	3-5	5+	Male	Female			
90-0142	Hopkins R.	6	3	3	0	0	0	6	42	0	0	-	0	0	0	0	0	0	6	12/04/90	Redfin perch
	Sub Total	6	3	3	0	0	0	6	42	0	0	-	0	0	0	0	0	0	6		
90-0175	Murray R. *	1	0	1	0	0	1	0	7	0	0	-	0	0	0	0	0	0	1	9/05/90	Redfin perch
91-0430	Murray R.*	1	0	1	0	0	0	1	7	0	0	-	0	0	0	0	0	0	0	25/10/91	Redfin perch
	Sub Total	2	0	2	0	0	1	2	14	2	0	0	0	0	0	0	0	0	1		
90-0246	L. Burrumbeet	15	3	12	0	9	0	15	90	0	10	-	0	0	0	10	1	9	-	7/06/90	Redfin perch
	Sub Total	15	3	12	0	9	0	15	90	0	10	-	0	0	0	10	0	9	-		
90-0374	L. Toolirook	6	0	6	0	0	0	6	42	0	1	-	0	0	0	1	0	1	-	20/08/90	Redfin perch
	Sub Total	6	0	6	0	0	0	6	42	0	1	-	0	0	0	1	0	1	-		
90-0389	Blackman R. Tas	10	1	9	1	0	0	0	70	-	0	-	0	0	0	0	0	0	0	3/09/90	Redfin perch
	Sub Total	10	1	9	1	0	0	0	70	-	0	-	0	0	0	0	0	0	0		
90-0395	Goulb. R. Lagoon	3	1	2	0	0	3	0	21	-	0	-	0	0	0	0	0	0	1	6/09/90	Redfin perch
90-0433	"	5	0	5	0	0	5	0	35	-	0	-	0	0	0	0	0	0	-	5/10/90	Redfin perch
90-0469	"	2	0	2	0	0	2	0	14	-	0	-	0	0	0	0	0	0	-	31/10/90	Redfin perch
90-0515	"	5	0	5	0	0	1	4	35	0	0	-	0	0	0	0	0	0	-	4/12/90	Redfin perch
91-0012	"	4	0	4	0	0	3	1	28	0	1	-	0	0	1	0	0	0	0	10/01/91	Redfin perch
91-0067	"	10	0	10	0	0	8	2	70	-	0	-	0	0	0	0	0	0	-	6/02/91	Redfin perch
91-0091	"	4	2	2	0	0	3	1	28	0	0	-	0	0	0	0	0	0	0	6/03/91	Redfin perch
	Sub Total	33	3	30	0	0	25	8	231	0	1	-	0	0	1	0	0	0	1		
90-0393	L. Makoan	4	2	2	0	0	2	2	28	-	0	-	0	0	0	0	0		0	6/09/90	Redfin
90-0467	"	3	3	3	0	0	1	2	21	-	2	-	0	0	1	1	0		2	3/10/90	Redfin
90-0516	"	6	5	5	0	0	3	3	42	0	1	-	0	0	1	0	0		2	4/12/90	(Not incl. 1 x Murray cod, 2 x Goldern perch) Redfin
91-0014	"	16	16	16	0	0	12	4	112	0	0	-	0	0	0	0	0		5	10/01/92	Redfin
91-0069	"	7	7	7	0	0	5	2	43	0	0	-	0	0	0	0	0		5	6/02/91	Redfin
91-0090	"	9	8	8	0	0	7	2	48	0	0	-	0	0	0	0	0		2	6/03/91	Redfin (Goldern perch x 1 not incl.)
90-0435	"	2	2	2	0	0	0	2	14	0	0	-	0	0	2	0	0		1	5/10/90	(1 x Goldern perch not incl.)
	Sub Total	47		43	0	0	30	17	308	0	3	-	0	0	3	1	0		17		

90-0394	L. Nill	2	1	1	0	0	0	2	14	-	0	-	0	0	0	0	0	0	0	6/09/90	Redfin perch
90-0435	L. Nill	6	0	6	0	0	2	4	42	-	2	0	0	0	1	1	0	2	1	5/10/90	Redfin perch
90-0468	L. Nill	14	2	12	0	0	3	11	98	-	1	-	0	0	1	0	0	1	0	31/10/90	Redfin perch
91-0013	L. Nill	10	1	9	0	0	5	5	70	0	0	-	0	0	0	0	0	0	3	10/01/91	Redfin perch
91-0068	L. Nill	16	1	15?	12	0	3	1	78	0	0	-	0	0	0	0	0	0	3	6/02/91	Redfin perch
91-0089	L. Nill	10	4	6	1	4	2	3	52	-	0	-	0	0	0	0	0	0	4	6/03/91	Redfin perch
90-0514	L. Nill	15	6	9	5	8	1	1	73	-	0	-	0	0	0	0	0	0	2	4/12/90	Redfin perch
	Sub Total	73	15	58	18	12	16	27	427	0	3	0	0	0	2	1	0	3	13	18/09/90	
90-0414	L. Mead Tas	8	4	4	0	5	3	0	56	-	0	-	0	0	0	0	0	0	-	18/09/90	Redfin perch
	Sub Total	8	4	4	0	5	3	0	56	0	0	0	0	0	0	0	0	0	0		
90-0448	L. Eildon	13	3	10	0	0	0	13	91	-	0	0	0	0	0	0	0	0	2	15/10/90	Redfin perch
90-0450	L. Eildon	23	6	17	0	0	0	23	161	-	9	-	0	0	0	9	4	5	3	22/10/90	Redfin perch
	Sub Total	36	9	27	0	0	0	36	252	0	9	0	0	0	0	9	4	5	5		
90-0513	Virus Tree site	7	0	7	0	0	1	6	42	-	0	-	0	0	0	0	0	0	-	4/12/90	Redfin perch
	Sub Total	7	0	7	0	0	1	6	42	0	0	0	0	0	0	0	0	0	0		
91-0431	Viscera ex. J.	3	?	?		0	?	?	?	12	0	0	-	0	0	0	0	0	0	0	25/10/90	ot including in final total due to unknown ages and sex.
	ex J. Langdon																				
	Sub Total	3	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0		
	Page Total	124	28	96	18	17	20	69	789	0	12	0	0	0	2	10	4	8	18		
	FINAL TOTAL	243	42	201	19	26	76	122	1086	0	27	0	0	0	5	22	5	22	43		

B. DIAGNOSTIC																																
														Analysis of individual tissues (ELISA)																			
SAN	Location	Animals	Male	Female	Age 0-1	Age 2-3	Age 3-5	Age 5+	Number of tissue	+ve virus isol.	+ve ELISA	+ ve path	B	L	S	G	I	AK	PK	B	L	S	G	I	AK	PK	+ve PCR	Date	Species
90-0006	L. Learmouth	4	-	-	-	-	-	-	20	3	3	0	4	4	4	0	0	4	4	3	3	3	0	0	3	3	2	3/01/90	Redfin perch
90-0018	Cope Cope L	1	-	-	-	-	-	-	4	0	1	1	1	1	1	0	0	1	1	1	1	1	0	0	1	1	-	12/01/90	Redfin perch
90-0033	Rubicon	3	-	-	-	-	-	-	12	-	3	-	0	3	3	0	0	3	3	0	3	3	0	0	3	3	1	22/01/90	Rainbow Trout
90-0048	Rubicon	13	-	-	-	-	-	-	26	-	0	1	13	0	13	0	0	0	0	0	0	0	0	0	0	0	1	1/02/90	Rainbow Trout
90-0079	L. Eildon	6	-	-	-	-	-	-	36	0	4	-	6	6	6	6	0	6	6	4	4	4	4	0	4	4	1	19/02/90	Redfin perch
90-0248	Snobs (Thornton)	4	-	-	-	-	-	-	24	-	0	-	4	4	4	4	0	4	3	0	0	0	0	0	0	0	1	13/06/90	Rainbow Trout
90-0258	Licola, McAlister	2	-	-	-	-	-	-	14	0	0	-	2	2	2	2	2	2	1	0	0	0	0	0	0	0	1	6/07/90	Rainbow Trout
90-0272	Blue Rock Dam	5	1	4	-	-	-	4	25	2	5	-	5	5	5	0	0	5	5	1	1	0	2	0	0	1	1	24/07/90	Rainbow Trout
90-0336	Triton, Tumut	3	-	-	-	-	-	-	6	-	0	-	0	0	2	0	0	2	2	0	0	0	0	0	0	0	-	6/08/90	Rainbow Trout
90-0429	?	2	-	-	-	-	-	-	14	0	1	-	2	2	2	2	2	2	2	0	0	0	0	0	0	1	-	3/10/90	Redfin perch
91-0019	?Rubicon	?	-	-	-	-	-	-	5	-	0	-	-	-	-	-	-	-	-	0	0	0	0	0	0	0	-	22/01/91	Atlantic salmon
91-0020	Mt Bold S.A.	3	-	-	-	-	-	-	3	3	3		0	3	0	0	0	0	0	0	3	0	0	0	0	0	3	22/01/91	Redfin perch
91-0118	L. Alexander S.A.	1	-	-	-	-	-	-	2	-	0	-	0	0	0	0	0	1	1	0	0	0	0	0	0	0	-	18/03/91	Redfin perch
91-0124	Snowy Mt.	23	-	-	-	-	-	-	69	-	4	-	0	0	23	0	0	23	23	0	0	4	0	0	4	4	5	21/03/91	Rainbow Trout
91-0146	W.A.	1	-	-	-	-	-	-	3	-	0	-	0	1	0	0	0	1	1	0	0	0	0	0	0	0	1	11/04/91	Rainbow Trout
91-0159	Bacchus Marsh	3	-	-	-	-	-	-	15	-	0	-	0	3	3	3	0	3	3	0	0	0	0	0	0	0	2	2/07/91	Redfin perch
91-0261	Adelaide S.A.	2	-	-	-	-	-	-	1	-	0	-	0	0	0	1	0	0	0	0	0	0	0	0	0	0	-	7/04/92	Rainbow Trout
92-0091	C. Winslow	4	-	-	-	-	-	-	16	4	12	-	0	4	4	0	0	4	4	0	4	4	0	0	4	4	1	11/12/90	Rainbow Trout
90-0528	?	1	-	-	-	-	-	-	6	-	0	-	1	1	1	0	0	1	1	0	0	0	0	0	0	0	-		Rainbow Trout
	FINAL TOTAL	81	1	4	0	0	0	4	301	12	36	2	38	39	73	18	4	62	60	9	19	19	6	0	19	21	20		

Fish Sera

Date	Source	No. Samples Tested Samples	Samples No.	% Inhib. at 1:10
7/11/91	R. Whittington	2	2 3	43.6 15.8
1-/4/91	P. Grant Nill, Mok, Goulb.	28	Mok (15) Goulb (25)	50.0 13.0
10/5/91	P. Grant Nill, Mok, Goulb	9	-	-
8/11/91	P. Grant, Nill, Mok, Goulb	16	-	-
6/12/91	P. Grant, Nill, Mok, Goulb	10	-	-
10/1/92	P. Grant, Nill, Mok, Goulb	10	Mok(5) Nil (1) Nil (6) -	38.0 39.0 37.0-
28/2/92	P. Grant, Nill, Mok, Goulb	14	Mok (2) Mok (4) Nilll (3) Nill (4)	34.0 80.0 29.0 61.0
12/3/92	P. Grant, Nill, Mok,Goulb	10	Nill (4)	37.5
8/4/92	P. Grant, Nill, Mok, Goulb	19	Mok (1) Mok (2) Mok (4)	64.0 48.0 64.0
12/5/92	P. Grant, Nill, Mok, Goulnb	8	Mok (1)	76/4
2/7/92	R. Whittington (4 other samples showed slight <10% inhibition)	14	91/65-25 92/26-13 91/3912-6	11.0 21.6 16.8

Table 3. Analyses of sera from redfin perch.

Comparison of the BIV proteins in cells and purified viruses (lane 4, A and B) revealed that specific proteins (e.g. 27-, 35-, 54-, 57-, 81- and 111kD) were not present in purified virus and may represent non-structural proteins. Conversely a 92kD protein was detected in purified BIV, but not amongst the intracellular proteins. The reason for this may reflect the amount of methionine in the protein and its comparative level amongst the cell-associated proteins or additional processing before incorporation into the virions.

The differences in polypeptide profiles between BIV and the EHNV isolates were more pronounced. The major differences were the presence of 99-, 92-, 61-, 49-, 43-, 36-, 30- and 26kD proteins in purified BIV. Some of these proteins may be analogous to the 101-, 88-, 60-, 51-, 35- and 29kD proteins from purified EHNV isolates. There did not appear to be any analogous EHNV proteins for the 43- or 26 kD proteins. The polypeptide profile of BIV also differed from that of the EHNV profiles in the apparent absence of proteins analogous to the 86-, 82-, 37- and 31kD proteins of EHNV.

Table 4. Size of EHNV and BIV calculated from intracellular arrays

	Size of $EHNV_{RF}$ in CHSE Cells (nm)	**Size of BIV in CHSE Cells (nm)**
Number of values	100	102
Mean	172	159
Standard deviation	±6	±6
T-value	14.708	
Degress of freedom	200	

The overall similarity of BIV-specific proteins led to the question "do these proteins react with antisera raised against EHNV?". Many of the BIV proteins reacted with antibodies against the piscine viruses; the EHNV and BIV profiles differed in the presence of a more intensely stained 112kD protein in BIV, the absence of many of the minor immuno-stained proteins (e.g. 76-, 61-, 42- and 38kD) and the absence of a major analogous 50kD protein when incubated with antiserum raised against purified $EHNV_T$. Similarly when EHNV proteins were incubated with polyclonal antiserum raised against BIV, cross reactions were again observed (Table 5). Proteins from uninfected CHSE-214 cells failed to react with the antisera used in these experiments as did infected cells with normal (control) sera. BIV also gave positive reactions via antigen-capture ELISAs and immunoelectron microscopy. Only one set of oligonucleotide primers were capable of differentiating between the piscine and amphibian iridoviruses. These results illustrate that at the ELISA and immunoelectron microscopical level it is not possible to differentiate between the different strains.

Comparison of restriction enzyme profiles for EHNV and BIV DNAs showed different patterns for digests with *Kpn*I, *Bam*III, *Hind*III and *Nco*I (Fig. 10) and for *Xba*I, *Bam*III and *Pst*I. Differences, however, between EHNV isolates were relatively minor. For *Kpn*I, *Bam*III and *Nco*I, whilst most bands were common to both $EHNV_T$ and $EHNV_{RF}$ DNA, some bands were present in only one of the EHNV isolates. HindIII fragment sizes were indistinguishable between EHNV isolates by conventional and field inversion gel electrophoresis.

Estimates of genome size by addition of restriction fragment sizes (using enzymes *KpnI, XbaI, BamHI, BglII, NcoI* and *HindIII)* indicated that the two isolates of EHNV had DNAs that were 12510kb and that of BIV was 10912kb.

Table 5. Representative ELISA optical densities (O.D.) at 450 nm for the various isolates captured by antibodies against $EHNV_T$ and $EHNV_{RF}$. O.D. values represent (sample - control (uninfected cells)).

1° Antibody	2° Antibody	OD $EHNV_{RF}$	OD $EHNV_T$	OD BIV
Sheep anti-$EHNV_T$ (whole virus)	rabbit anti-$EHNV_T$ (whole virus)	1.53	1.32	0.74
	rabbit anti-$EHNV_T$ (capsid)	1.43	1.74	0.74
Sheep rabbit anti-$EHNV_{RF}$ (whole virus)	rabbit anti-$EHNV_{RF}$(whole virus)	1.11	1.38	
	rabbit anti-BIV (whole virus)	1.65	1.75	1.13
	rabbit anti-$EHNV_T$(whole virus)			1.12
	rabbit anti-$EHNV_T$(capsid)			1.27
	rabbit anti-$EHNV_{RF}$ (whole virus)			0.97
	rabbit anti-BIV(whole virus)			1.47

A cross-hybridization experiment using approximately equal concentrations of DNA from all three iridovirus isolates with vaccinia virus and calf thymus DNA as controls, indicated a high level of sequence homology between the three iridoviruses. No cross reactions with either vaccinia virus or calf thymus DNA were observed. The high stringency conditions used to wash the blots suggested a level of homology in the order of 90%. In each case a slightly higher level of hybridization, indicated by the intensity of the spots, was achieved with the homologous probe. These results indicate that EHNV and BIV are closely related and should be grouped within the one genus.

Diagnosis of exotic viruses

The final objective listed for this project was to develop diagnostic tests for infectious pancreatic necrosis virus (IPNV), infectious haematopoietic necrosis virus (IPNV) and viral haemorrhagic septicaemia virus (VHSV).

Since the commencement of this project, the Australian Fish Health Reference Laboratory (AFHRL) was relocated to, and assimilated with, AAHL. The relocation of this laboratory permitted the functions and capabilities of AFHRL to be enhanced. Part of AFHRL's new mission was to develop diagnostic tests for IPNV, IHNV and VHSV. As these objectives were already encompassed within the auspices of this project, it was decided that AFHRL would concentrate on the generation of antibodies to IHNV and VHSV and we would concentrate on the developement of ELISA's and immunoelectron microscopy for the detection of IPN. The results from this work are described below.

Antibodies to the Ab and Sp strains of IPNV were generated. Antisera were generated in both rabbits and guinea pigs. An antigen capture ELISA was developed as was immunoelectron microscopical procedures for both strains. The antisera were found to possess neutralising capabilities and were (a) of high titer and (b) capable of differentiating between different strains of IPNV. The antisera and ELISA protocols have been given to AFHRL where they have been tested against their reference stocks of IPNV. The antisera have subsequently been aliquoted and stored away at -80°C for future use.

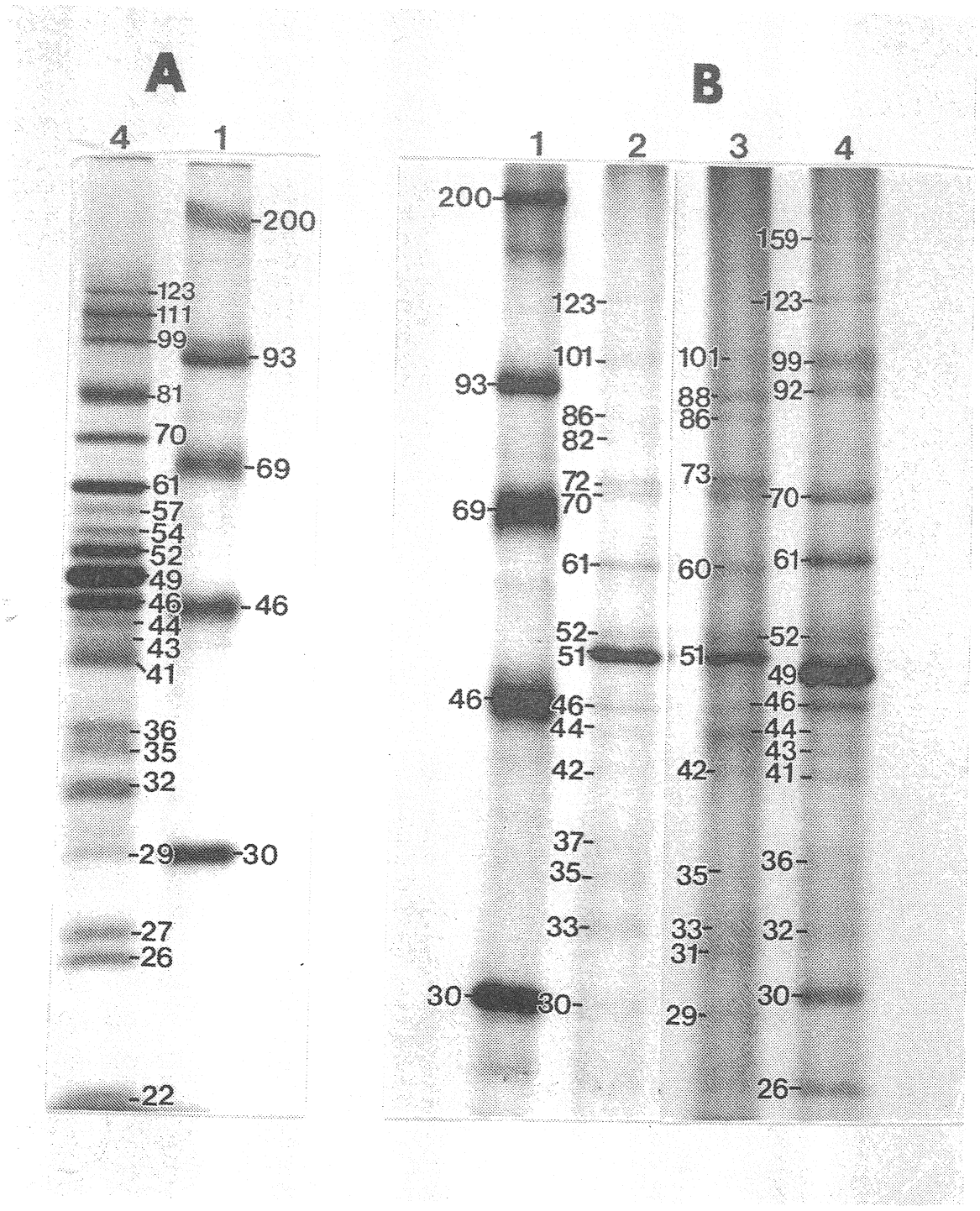

Fig 9 Proteins in (A) BIV-infected cells and (B) purified ENV_T, $EHNV_{RF}$ and BIV. Radioactive labelled proteins in lanes 1 to 4 were detected by autoradiography. The estimated molecular weight are given in kD. Lane 1, markers; lane 2, EHNVT, lane 3, EHNVRF and lane 4, BIV.

Significance of results

EHNV$_T$ and EHNV$_{RF}$ differ at the protein and genome level

It is now possible to differentiate between two strains of EHNV. These strains, however, are only two of many which have been isolated in Victoria. It is therefore necessary to plaque purify and generate stocks of the other isolates if valid comparisons are to be made. This requirement is further illustrated by the isolation of infectious EHNV and positive antisera to EHNV from healthy redfin perch. These results indicate that redfin may survive epizootics of EHNV infection and become carriers. The presence of positive antisera in apparently healthy fish further supports the suggestion that redfin perch may survive virulent EHNV infections. Alternatively, the results may be interpreted as follows. In the field there are many serotypes of circulating EHNV, some of which are capable of causing disease (i.e. they are virulent) and others not. The detection of antigen and antisera to EHNV, in apparently healthy redfin perch, may be indicative of non-virulent forms of the virus circulating in the wild. The finding of positive antisera to EHNV in healthy fish is of particular importance as it shows that antisera can survive in wild animals between the seasons when EHN disease occurs and therefore a long term strategy for vaccination of commercial stock may be worth pursuing. Overall, the results show that further work must be performed to elucidate the diversity of EHNV present in the water-systems of Victoria and indeed N.S.W. particularly in terms of virulence to both redfin perch and rainbow trout. The results from this work would be important for the classification of EHNV (virulent) free areas and/or farms.

To date it is only possible to differentiate between EHNV strains by analysing the constituent proteins by polyacrylamide gel electrophoresis and western blot analyses. The polyclonal antisera and PCR cannot differentiate between the strains. This is not surprising as (a) polyclonal antisera contains antibodies against numerous epitopes(proteins) of which many are shared between isolates and (b) to generate discriminatory oligonuceotides the entire DNA sequence would have to be known for both representatives serotypes; this information is at present not known. It should also be noted that the antibodies cannot discriminate between EHNV and BIV at the level of the antigen-capture ELISA; it is therefore important to generate monoclonal antibodies which could in-turn be used to discriminate between the piscine iridoviruses and between the EHNV and BIV iridoviruses. The generation of several oligonucleotides have however resulted in the development of discrimatory PCR assays (EHNV versus BIV only). The success of this part of the project means that non-destructive sampling can be implemented for screening of expensive brooding and/or commercial stock.

Diagnostic tests for EHNV can now be performed quickly and reliably for a diverse range of tissues

The antibodies generated in this study to EHNV have proved to be of high quality. They have been used to detect EHNV by light microscopy, electron microscopy, western blot analyses, antigen-capture ELISA and antibody-competitive ELISA's. The above assays were tested on tissue culture material, diagnostic and survey samples. The resultant data confirmed that liver, spleen and kidney were the optimum tissues for assay and that the tests were valid even when the diagnostic samples were badly decomposed. Routine diagnostic procedures such as histopathology and negative contrast electron microscopy in State Laboratories can now be updated to include the labelling of viruses with antibodies and associated markers (e.g. colloidal gold and peroxidase).

PCR was also successfully developed for EHNV detection. Experimental results showed that whilst this is an exquisitely sensitive test, the people involved must be aware of the inherent problems. The problems originate from the sensitivity of the test itself. It is crucially important that investigators use decominated equipment and are scrupiously clean in their sample preparation. To overcome any possible contamination of our samples each sample was preceded and followed by

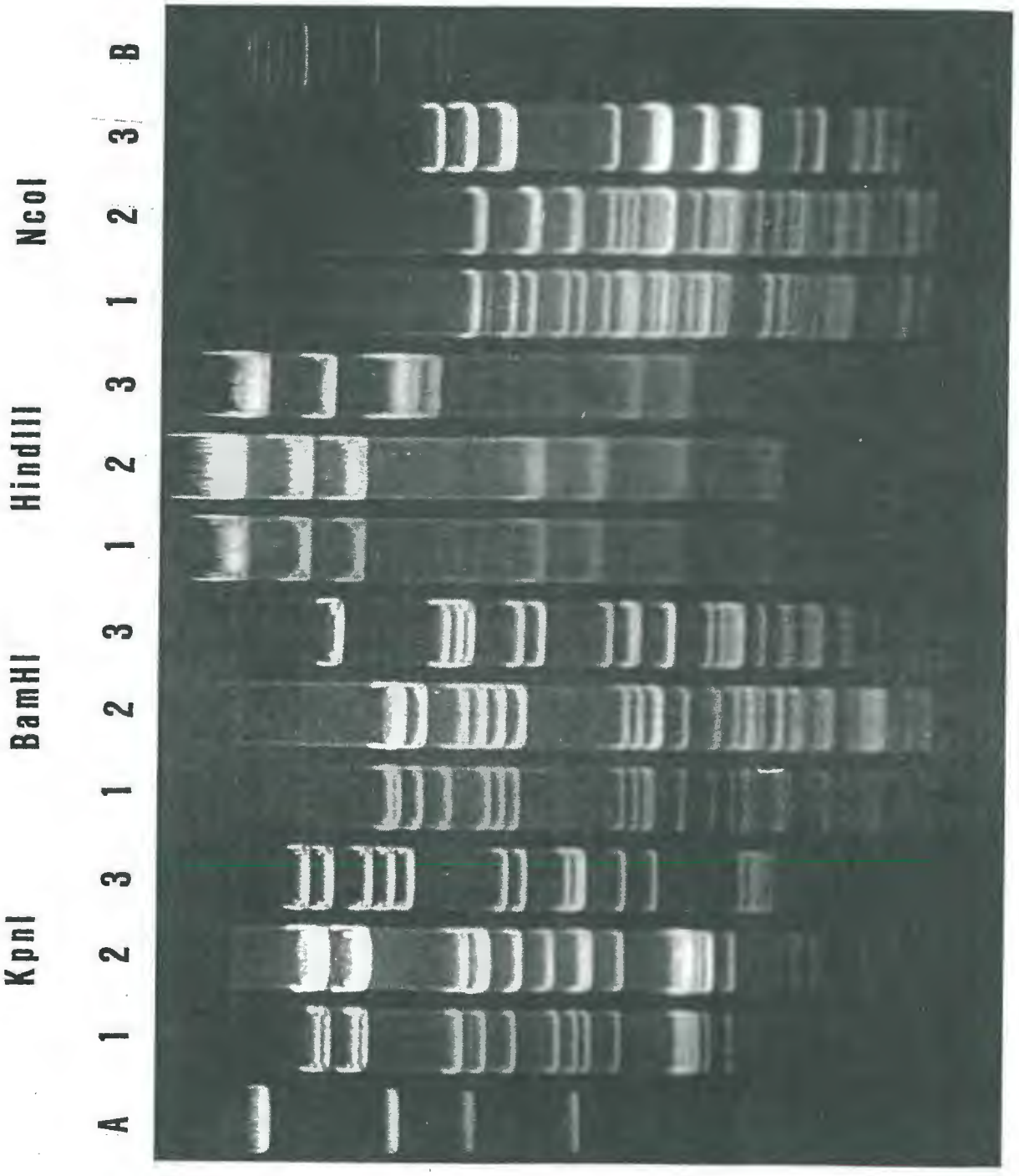

Fig. 10 Restriction endonuclease digestion patterns for the DNA from (1) $EHNV_{RF}$, (2) $EHNV_{T}$, and (3) BIV digested with *Kpn*I, *Bam*III, *Hind*III or *Nco*I. Markers were A DNA digested with *Hind*III (23.1, 9.42, 6.68, 4.36, 2.32 and 2.03 kb); and B high molecular weight markers (48.5, 38.4, 33.5, 29.9, 24.8, 22.6, 19.4, 17.1, 15.0, 12.2, 10.1, 8.6 and 8.3 kb).

suitable controls; therefore if either of these in-built controls were positive then the sample was discarded and the entire test and laboratory surrounds including equipment decontaminated. If this operating system is implemented then the test works to specifications. Using this test we detected EHNV in survey samples where EHNV was not detected by any other means. These results were surprising in that they showed that EHNV DNA was present at extremely low levels in redfin perch throughout all water basins sampled. On the positive side however (as stated above) the test can be used to screen important (e.g. financial and/or politically sensitive) samples. The detailed results from this and the sampling work will be published soon.

The production of antigen-capture ELISA's at both AAHL and the EMAI now means that both tests can be compared and the optimum parts of each combined to produce a high quality ELISA which should, over time, be accepted as the standard test for EHNV detection. Further work should also be pursued whereby the supplied antigen is inactivated and the detection antibodies are eventually replaced with discrimatory monoclonal antibodies. The antigen-capture ELISA and other developed technologies will be available to State Laboratories within the next 12 to 24 months. The PCR technology will remain "in-house" but will be offered as a service to interested "bodies".

Where did EHNV come from and where can it go?

It could be argued that EHNV is ubiquitous throughout southern Australia and its rise to prominence is due to the introduction of susceptible sporting fish such as the redfin perch. It is difficult however to explain why it took so long for the epizootics to be observed as redfin perch were introduced into Australia in 1868. It is possible that EHNV has been introduced into this country in the past three to four decades (based on recorded and anecdotal evidence of large fish kills). If this is true, then the virus must have been introduced by fish other than redfin perch or perhaps by another host.

During the course of the study we also studied BIV from Queensland (refer above for details). This virus has a striking resemblance to EHNV and FV3. Furthermore, (a) recent work by Hendrick *et al.*, (1992) confirmed that EHNV resembles FV3 and (b) correspondence with scientists in Venezuela have indicated that iridoviruses isolated from the canetoad *bufo marinus* L. react with antisera raised against EHNV. These results have shown (Hendrick *et al.*, (1992); Hengstberger *et al.*, (1992)) that EHNV and the North American ranaviruses are members of the same genus. The ability of BIV to infect and kill freshwater fish has recently been demonstrated by Dr R. Speare (personal communication); these results therefore show that BIV can cross the species barrier. It is therefore possible that EHNV may have evolved from an amphibian isolate. The potential for both BIV to infect a range of fish and EHNV to infect frogs and toads needs to be performed in the near future as it is possible that the spread of EHNV and possibly other potentially lethal irido-associated viruses may be mediated by the spread of amphibians, in particular cane toads.

Detection of exotic viruses

The collaboration of scientists employed by this project and AFHRL has culminated in the production of antisera to a range of viruses including IPNV, IHNV and VHSV. Work performed under this project successfully generated antisera against IPNV. The antisera was subsequently used to generate antigen capture ELISA's and immunoelectron microscopical techniques. The antisera, tests and procedures have been transferred to AFHRL where they now form part of their diagnostic armouridium.

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