Experimental infection of Australian anurans (*Limnodynastes terraereginae* and *Litoria latopalmata*) with Bohle iridovirus

B. R. Cullen^{1,*}, L. Owens¹, R. J. Whittington²

¹Department of Biomedical and Tropical Veterinary Sciences, James Cook University of North Queensland, Townsville, Queensland 4811, Australia

²Elizabeth Macarther Agricultural Institute, Private Bag No. 8, Camden 2570, Australia

ABSTRACT: Challenge studies with Bohle iridovirus (BIV), a recently isolated Australian iridovirus, were performed with 2 Australian anurans. Juvenile *Limnodynastes terraereginae* and *Litoria latopalmata* were highly susceptible to BIV, while larval *L. terraereginae* were less susceptible. *L. terraereginae* and *L. latopalmata* juveniles injected intraperitoneally with 10^3 TCID₅₀ (50% tissue culture infective dose) ml⁻¹ BIV died 6 to 10 d after injection. BIV could be transmitted by natural routes in *L. terraereginae* with mortality occurring from Day 14 to 26. Mortality occurred on Days 13 and 33 in *L. terraereginae* juveniles that were bath-challenged with BIV at a titre of 10^3 TCID₅₀ ml⁻¹. BIV was retained for at least 40 d in the frogs. Larval *L. terraereginae* were bath-challenged at 10^6 , 10^1 , $10^{2.5}$ and 10^4 TCID₅₀ ml⁻¹ BIV. Low mortality occurred in the $10^{2.5}$ and 10^4 TCID₅₀ ml⁻¹ challenges, but BIV was isolated from one tadpole in the 10^1 TCID₅₀ ml⁻¹ treatment sacrificed 16 d post challenge. Pathological changes were similar for larvae and juveniles of both species, and included renal, hepatic, splenic and pulmonary necroses, with pyknosis of erythrocytes also being observed. Immunoperoxidase staining techniques using cross-reactive anti-epizootic haematopoietic necrosis virus (EHNV) polyclonal antibody indicated the presence of BIV in the kidney, liver, lung and spleen of infected juveniles.

KEY WORDS: Bohle iridovirus · Anuran · Ranavirus · Amphibian decline

INTRODUCTION

Bohle iridovirus (BIV) was isolated from metamorphs of the ornate burrowing frog *Limnodynastes ornatus* collected at a temporary pond near Townsville, Australia. It caused mortalities in these captive amphibians during or soon after metamorphosis (Speare & Smith 1992). BIV was also pathogenic to adults of the introduced species *Bufo marinus*. Infection resulted in an often fatal systemic disease, with hepatic, pulmonary and renal haemorrhages and necroses being observed (authors' unpubl. data). BIV infection in *B. marinus* was induced by subcutaneous injection or consumption of contaminated food, and could be spread by cohabitation.

BIV is the only virus found to be pathogenic to both amphibians and fish. Infection of barramundi *Lates calcarifer* with BIV resulted in 100% mortality, with focal to diffuse necrosis of the haematopoietic tissue of the kidney and spleen being characteristic (Moody & Owens 1994).

The first and only other virus isolated in Australia from vertebrate poikilotherms was epizootic haematopoietic necrosis virus (EHNV) in redfin perch *Perca fluviatilis* in Victoria (Langdon et al. 1986). BIV and EHNV are closely related but distinct iridoviruses, with affinities to the *Ranavirus* genus (Eaton et al. 1991, Hyatt et al. 1991, Speare & Smith 1992, Hengstberger et al. 1993). The 2 iridoviruses were similar morphologically, ultrastructurally, serologically and in aspects of viral morphogenesis. They also caused cytopathic effect in a similar range of cell lines. Differences were noted in size, polypeptide profiles, cytopathic

*E-mail: brandford.cullen@jcu.edu.au

effect and restriction endonuclease digest profiles of their respective DNAs (Hengstberger et al. 1993). Differences in host susceptibility have also been demonstrated. Barramundi were susceptible to BIV (Moody & Owens 1994) but refractory to EHNIV, whilst rainbow trout *Oncorhynchus mykiss* were susceptible to EHNIV (Langdon et al. 1988, Langdon 1989) but refractory to BIV (Speare pers. comm.).

Apart from the initial outbreak, it was unknown whether BIV caused disease in native Australian amphibians. This study investigated the susceptibility of 2 native frog species, *Limnodynastes terraereginae* tadpoles and juveniles, and *Litoria latopalmata* juveniles, to BIV.

MATERIALS AND METHODS

Capture and husbandry of amphibians. *Limnodynastes terraereginae* and *Litoria latopalmata* tadpoles were captured with dipnets from a freshwater creek on a Mount Spec cattle station (146° 01' E, 19° 10' S). After transportation to the laboratory, tadpoles were removed from the creek water and placed in a glass tank of 223 l capacity containing tap water. Previously, the glass lids were placed in the tank which was filled with tap water to which sodium hyperchlorite was added (>30 ppm active chlorine), and left standing for at least 24 h. All aquarium equipment and other housing containers were chlorinated similarly. The tank was then drained, rinsed and filled with normal tap water and aerated (Takatsuki Electric, Japan) overnight to remove any residual chlorine. Upon introduction of the tadpoles, the tank was aerated continuously. A power filter (Fluval 403, Rolf C. Hagan, Montreal, PQ, Canada) filled with porcelain particles, activated carbon and filter wool recirculated and filtered the water at a rate of 3.8 total water volume turnovers per hour. Bricks were placed in the centre of the tank so as to rise just above the water level. This was provided to allow metamorphosing frogs to escape from the water. Tadpoles in the main holding tank were fed upon lettuce which was boiled for 5 min, and flake fish food (Wardley's Tropical Flake, Secaucus, NJ, USA) was also provided. A water change of approximately one third was carried out every 2 to 3 d. Faeces were also siphoned off at this time. Newly metamorphosed frogs were removed and placed in a dry glass tank 95 cm length × 60 cm width × 40 cm height. A plastic petri dish (Disposable Products, Adelaide, Australia) filled with tap water was also provided, with the water being changed daily. Metamorphs were fed every few days upon termites collected on the James Cook University campus. Once a week, metamorphs were removed from the tank and

placed in a clean plastic bucket to allow the tank to be cleaned. This involved wiping the bottom of the tank with a paper towel so as to remove all faeces. The bottom of the tank was kept dry at all times by wiping any water spillage with a paper towel.

Maintenance of amphibians under experimental conditions. Rectangular plastic containers (30 × 24 × 12 cm) with insect screen lids of 3 mm mesh were used to house experimental tadpoles. Tadpoles were fed upon boiled lettuce and flake fish food daily. Experimental containers were aerated continuously and a daily water change of approximately one half was carried out commencing 2 d after viral challenge. Water temperature ranged from 23 to 28°C.

Metamorphs of each treatment were housed in plastic containers (30 × 24 × 12 cm) with insect screen lids (3 mm mesh). A plastic petri dish filled with distilled water was provided. Metamorphs were fed every 2 or 3 d upon termites collected on the James Cook University campus. Air temperature ranged from 18 to 27°C throughout the *Limnodynastes terraereginae* experiment, and from 23 to 28°C throughout the *Litoria latopalmata* experiment.

Culture of BF2 cells. Cultures of bluegill fry (BF2) (Wolf et al. 1966) cells of passages 77 to 121 were used for viral propagation titration. Cell monolayers were grown in 150 cm² plastic culture flasks (Corning Glass Works, Corning, NY, USA) containing Dulbeccos' Modified Eagles' Medium (DMEM) plus 5% foetal bovine serum (FBS). The medium was buffered with sodium bicarbonate to maintain a pH of 7 to 8. Flasks were incubated at 26°C.

Culture of stock BIV. BIV, originally isolated from the ornate burrowing frog *Limnodynastes ornatus* (Speare & Smith 1992) and then passaged through a barramundi *Lates calcarifer*, was used. Cell medium from a 150 cm² BF2 cell culture flask was discarded and approximately 2 ml of 10⁵ TCID₅₀ (50% tissue culture infective dose) ml⁻¹ BIV was introduced. The flask was then left to stand for 30 min. New cell medium consisting of DMEM plus 5% FBS was subsequently added, and the flask was incubated at 26°C. When the cell monolayer was completely destroyed by BIV (24 h), the medium was frozen and thawed 3 times and stored at -66°C.

The viral titre of the stock BIV was determined to be 10⁵ TCID₅₀ ml⁻¹ as calculated by the method of Reed & Muench (1938). Dilutions of stock BIV were made in DMEM for inoculation experiments and in water for bath exposure experiments.

Bath exposure of *Limnodynastes terraereginae* tadpoles to BIV. Tadpoles were challenged at the following viral concentrations: 5 tadpoles at 10⁰ TCID₅₀ ml⁻¹ BIV, 5 tadpoles at 10¹ TCID₅₀ ml⁻¹ BIV, 10 tadpoles at 10^{2.5} TCID₅₀ ml⁻¹ BIV and 5 tadpoles at 10⁴ TCID₅₀ ml⁻¹

BIV; 10 tadpoles were left as uninfected controls. Weights of tadpoles ranged from 0.52 to 2.87 g.

Tadpoles randomly chosen from the main holding tank were added to the experimental containers. For the 10^0 , 10^1 and $10^{2.5}$ TCID₅₀ ml⁻¹ BIV challenges, the appropriate amount of stock virus was added to the containers to achieve the desired concentration. For the 10^4 TCID₅₀ ml⁻¹ challenge, tadpoles were placed in a container of 150 ml volume of the appropriate viral concentration for 1 h. The tadpoles and the viral fluid were then placed into the 5.6 l plastic container.

Unless mortality occurred, 1 individual in the 10^0 , 10^1 and 10^4 TCID₅₀ ml⁻¹ bath treatments, and 2 individuals in the control and $10^{2.5}$ TCID₅₀ ml⁻¹ bath treatments, were sacrificed with chloroform every 2 d commencing 6 d post-challenge. The dead tadpoles were cut in half longitudinally with a sterile scalpel, with half the tadpole prepared for viral isolation and the other half for histology.

Infection of *Limnodynastes terraereginae* metamorphs with BIV. Eight metamorphs randomly chosen from the main holding tank were placed in each of the following treatments: 10^3 TCID₅₀ ml⁻¹ BIV bath challenge; 10^3 TCID₅₀ ml⁻¹ injection challenge; frogs placed 'in contact' with the injected frogs and controls. Weights of metamorphs ranged from 0.56 to 3.39 g.

For the bath BIV challenge, stock BIV was added to the distilled water in the petri dish to obtain a 10^3 TCID₅₀ ml⁻¹ challenge. For the injection treatment, a dose of 0.1 ml of 10^3 TCID₅₀ ml⁻¹ BIV was injected intraperitoneally in the ventral surface just to one side of the midline of each frog using a 25G × 5/8" needle on a 1 ml syringe. A new needle and syringe were used for each individual. For identification, the 'in contact' frogs had the fifth toe of the right hind leg removed with a sterile scalpel, and they were then placed into the container housing the injected frogs. Control frogs were placed in containers identical to those for the BIV challenged frogs.

Unless mortality occurred, 1 frog per treatment was sacrificed every 5 d. Dead frogs were cut in half longitudinally with a sterile scalpel, with one half prepared for viral isolation and the other for histology.

Infection of *Litoria latopalmata* metamorphs with BIV. Only 3 metamorphs were available for experimentation; 2 of these were challenged with 10^3 TCID₅₀ ml⁻¹ BIV intraperitoneal injection, and 1 frog acted as a control. Weights ranged from 1.38 to 2.16 g. These metamorphs were infected, housed and treated in a manner identical to the *Limnodynastes terraereginae* metamorphs.

Histological preparation. The longitudinally halved tadpoles/metamorphs for histological examination

were fixed in Bouin's fixative for at least 48 h, washed in 70% ethanol, embedded in paraffin and sectioned at a thickness of 5 µm. Tissues were stained with Mayer's haematoxylin and eosin (H&E), Prussian blue and Schmorl's stain, and observed under light microscopy.

Viral isolation. The longitudinally halved tadpoles/metamorphs for viral isolation were placed into individual stomacher bags (Seward Medical, London). Approximately 1 ml of DMEM was added to the bag and the contents homogenised. The liquid was removed, frozen and thawed 3 times and clarified by centrifugation at $7240 \times g$ for 15 min. Two hundred µl of supernatant was added to BF2 cell monolayers in 24-well flat-bottomed culture plates (Corning Glass Works) which were incubated at 26°C. The plates were observed for 14 d for the onset of characteristic BIV cytopathic effect (CPE) (Speare & Smith 1992).

Immunoperoxidase staining of histological sections. Immunoperoxidase staining was carried out on sections of 2 control *Limnodynastes terraereginae* metamorphs which contained all relevant tissues, and all metamorphs in the bath, injection and 'in contact' viral challenge treatments. All 3 experimental *Litoria latopalmata* metamorphs were also stained. Since BIV and EHNIV cross-react to a high degree serologically (Hengstberger et al. 1993), an anti-EHNIV based system was used. Affinity-purified rabbit anti-EHNIV primary antibody with affinity-purified goat anti-rabbit conjugate was used in immunoperoxidase staining. Dilutions of 1:1000 of both primary antibody and conjugate were used with immunoperoxidase staining.

The tissues embedded in paraffin were cut to a thickness of 5 µm, placed on a glass slide, incubated at 60°C for 12 h and dewaxed in xylene and absolute ethanol. Sections were bathed in a solution of 1% hydrogen peroxide in phosphate buffered saline (PBS) for 10 to 15 min at room temperature, and were then washed 3 times for 5 min in TEN-Tw (0.05 M Tris, 0.3 M NaCl, 0.0004 M ethylenediaminetetraacetic acid, 0.05% Tween 20) buffer. Sections were then bathed in primary antibody for 1 h at room temperature. After three 5 min washes in TEN-Tw buffer, the sections were bathed in conjugate for 1 h at room temperature. Then, after another three 5 min washes in TEN-Tw buffer, sections were bathed in substrate solution for 30 min at room temperature. The substrate consisted of 10 ml of acetate buffer (pH 5), 500 µl of a solution consisting of 20 mg of 3-amino-9-ethylcarbazole dissolved in 5 ml of N,N-dimethylformamide, and 5 µl of 30% hydrogen peroxide solution. After three more 5 min washes in TEN-Tw, slides were counterstained with Mayer's haematoxylin and mounted.

RESULTS

Bath challenge of *Limnodynastes terraereginae* tadpoles with BIV

No mortality or pathological changes were observed in any of the tadpoles in the control, 10^0 and 10^1 TCID₅₀ ml⁻¹ BIV bath challenges. BIV was isolated from 1 tadpole in the 10^1 TCID₅₀ ml⁻¹ challenge sacrificed 16 d post-challenge.

In the $10^{2.5}$ TCID₅₀ ml⁻¹ BIV challenge, 2 tadpoles died 6 d post-challenge and 1 died 11 d post-challenge. Pathological changes were observed in the tissues of the 3 tadpoles which died, but BIV could not be isolated in BF2 cell culture.

In the 10^4 TCID₅₀ ml⁻¹ BIV challenge, 1 tadpole died 7 d post-challenge, but BIV was not isolated. BIV was isolated from 1 tadpole sacrificed 16 d post-challenge and 1 sacrificed 18 d post-challenge, but no pathological changes were observed in the tissues of any individuals.

Table 1. Occurrence of mortality, pathological lesions, viral isolation and specific immunoperoxidase (IP) staining of *Limnodynastes terraereginae* metamorphs challenged with Bohle iridovirus (BIV)

Infection treatment	Specimen	BIV isolated	Pathological lesions	IP stain
Control	Died Day 5	—	—	—
	Died Day 6	—	—	—
	Sacrificed Day 15	—	—	—
	Sacrificed Day 20	—	—	—
	Sacrificed Day 25	—	—	—
	Sacrificed Day 30	—	—	—
	Sacrificed Day 35	—	—	—
Bath	Sacrificed Day 40	—	—	—
	Sacrificed Day 5	—	—	—
	Sacrificed Day 10	+	—	—
	Died Day 13	+	+	+
	Sacrificed Day 20	+	—	—
	Sacrificed Day 25	+	—	—
	Sacrificed Day 30	—	—	—
Injection	Died Day 33	+	+	+
	Sacrificed Day 40	—	—	—
	Sacrificed Day 5	+	—	—
	Died Day 6	—	+	+
	Died Day 7	+	+	+
	Died Day 7	+	+	+
	Died Day 8	—	+	—
In contact	Died Day 9	—	—	—
	Died Day 9	+	+	+
	Died Day 10	—	+	+
	Sacrificed Day 5	—	—	—
	Sacrificed Day 10	—	—	—
	Died Day 14	+	+	+
	Died Day 15	+	+	+
	Died Day 21	+	+	+
	Died Day 26	—	+	+
	Sacrificed Day 35	—	—	—
	Sacrificed Day 40	+	—	—

Limnodynastes terraereginae metamorphs challenged with BIV

One control metamorph died on each of Days 5 and 6 after commencement of the experiment (Table 1). BIV could not be isolated from these individuals and no BIV-associated pathological changes were apparent.

In the bath exposure, a metamorph died on each of Days 13 and 33 post-challenge, with pathological changes evident in both (Table 1). BIV could be isolated from both metamorphs which died, and from 3 other individuals which were sacrificed (Table 1).

Of 8 metamorphs challenged with BIV via intraperitoneal injection, 7 died and 1 was sacrificed 5 d post-challenge (Table 1). Mortality occurred 6 to 10 d post-challenge, with all but 1 individual displaying pathological changes. BIV was isolated from 3 individuals (Table 1).

Four 'in contact' metamorphs died 14 to 26 d after commencement of the experiment, and all had pathological changes in their tissues. Mortality of frogs in the 'in contact' treatment thus began 4 d after the death of the last frog of the injection treatment. BIV was isolated from 3 of the 4 metamorphs which died, and from 1 metamorph sacrificed 40 d after commencement of the experiment (Table 1).

Injection of *Litoria latopalmata* metamorphs with BIV

The 2 BIV-injected *Litoria latopalmata* metamorphs died 8 and 10 d post-challenge. Pathological changes were observed upon histological examination, and BIV was isolated from the tissues of both these frogs. The control frog was sacrificed at Day 10, with no pathological changes or viral isolation occurring.

Histological findings

No obvious external manifestations of disease were observed. Where present, pathological lesions were similar for *Limnodynastes terraereginae* tadpoles and metamorphs, and *Litoria latopalmata* metamorphs. Focal to extensive necroses were observed in tissues of the kidney, liver, stomach, spleen and lung. No pathological changes were observed in any control tadpoles or metamorphs (Figs. 1, 4, 6 & 8).

Kidney. Melanisation was common throughout the glomeruli and tubules (Fig. 2). Interstitial haemorrhaging ranged from slight to extensive.

Fig. 1. *Limnodynastes terraereginae*. Kidney from a control individual. Glomeruli (arrows) and tubules (arrowheads). H&E. Scale bar = 24 μ m. Fig. 2. *Limnodynastes terraereginae*. Kidney from an individual infected with *Bole iridovirus* showing melanisation (arrow) and extensive necrosis of tubules (arrowhead). H&E. Scale bar = 24 μ m. Fig. 3. *Litoria latopalmata*. Kidney from an individual infected with *Bole iridovirus* showing shrunken and necrotic glomeruli (arrowheads), pyknosis of glomerular (small arrow) and tubular (large arrows) cell nuclei. H&E. Scale bar = 24 μ m. Fig. 4. *Limnodynastes terraereginae*. Liver from a control individual. Hepatic cells and erythrocytes (arrow). H&E. Scale bar = 24 μ m

Fig. 5. *Limnodynastes terraereginae*. Liver from an individual infected with *Bohle iridovirus* showing necrosis, pyknosis of hepatic cells (arrows) and melanomacrophage centres (arrowhead). H&E. Scale bar = 24 µm. Fig. 6. *Limnodynastes terraereginae*. Spleen from a control individual. Splenic pulp and erythrocytes (arrowhead). H&E. Scale bar = 19 µm. Fig. 7. *Limnodynastes terraereginae*. Spleen from an individual infected with *Bohle iridovirus* showing extensive necrosis of splenic pulp, pyknosis (arrowheads) and melanisation (arrow). H&E. Scale bar = 19 µm. Fig. 8. *Limnodynastes terraereginae*. Stomach from a control individual showing gastric glands. H&E. Scale bar = 24 µm

Fig. 9. *Limnodynastes terraereginae*. Stomach from an individual infected with Bohle iridovirus showing generalised necrosis of mucosa resulting in loss of structural architecture, and pyknosis (arrows). H&E. Scale bar = 24 μ m

Tubule damage ranged from focal to locally extensive and included cytoplasmic degeneration, pyknosis of nuclei, chromatin margination within nuclei, lysis of nuclei and generalised necrosis (Fig. 2). Necrosis also occurred in the interstitial haematopoietic tissue. Glomeruli were often shrunken and contained pyknotic nuclei (Fig. 3).

Liver. An increase in the numbers and size of melanomacrophage centres accompanied by diffuse melanisation was apparent throughout the liver. The liver was often congested and many erythrocytes were pyknotic. Hepatocellular pyknosis and locally extensive necrosis were also observed (Fig. 5).

Spleen. Pyknosis and locally extensive to diffuse necrosis were apparent in splenic white pulp, particularly in haematopoietic tissue. Melanisation of the splenic pulp and pyknosis of erythrocytes were also observed (Fig. 7).

Stomach. In the stomach there was necrosis of the mucosa, gastric glands and to a lesser extent the underlying muscularis. There was melanisation, margination of nuclear chromatin and pyknosis in gastric glandular epithelia (Fig. 9).

Lung. Non-suppurative inflammation, focal necrosis and dystrophic calcification were observed in alveolar walls of infected frogs. Extensive linearly distributed melanisation was observed between smooth muscle and epithelia of bronchioles, but this was also observed in control frogs.

In addition, there was evidence of necrosis in the brain and spinal cord of some of the metamorphs and tadpoles which had died, with the nuclei of neurones and glial cells either displaying margination of chromatin or pyknosis.

Immunoperoxidase staining of *Limnodynastes terraereginae* metamorphs

The occurrence of specific staining in metamorphs was observed in all but 1 individual with pathological lesions (Table 1). Five metamorphs which lacked pathological lesions but from which BIV was isolated failed to show specific staining (Table 1).

Specific staining was observed in the liver, kidney and lung of some infected frogs of each treatment. These tissues in control frogs were either devoid of reactivity or contained a minimal amount of faint, generalised background stain.

Specific staining of the liver ranged from small, scattered foci to a large number of extensive focal areas.

Specific staining of the kidney was often concentrated in necrotic, shrunken glomeruli and interstitial haematopoietic tissue. Specific staining also occurred in some areas of both necrotic and normal tubules.

Specific staining occurred in the lung and spleen of some infected frogs; however, splenic tissue of control frogs was lost during the staining procedure, and was therefore not available for comparison. Specific staining was not observed in the brain and spinal cord.

Immunoperoxidase staining of *Litoria latopalmata* metamorphs

Staining of the liver, kidney and lung of the BIV-injected frogs was similar to that of the infected *Limnodynastes terraereginae* metamorphs, while no staining occurred in the control.

DISCUSSION

Both metamorphs and larvae of *Limnodynastes terraereginae* appeared to be susceptible to BIV, with infection by intraperitoneal injection, bath and 'in contact' methods resulting in systemic disease. Juvenile *Litoria latopalmata* were also susceptible to BIV, although only 3 individuals were examined. Mortality in this species occurred over a similar time scale, and histopathological changes were similar to those encountered in infected *L. terraereginae* juveniles. This extends the list of hosts susceptible to BIV-induced systemic disease to include a species of a third amphibian genus (*Litoria*), the other two being *Bufo* and *Limnodynastes* (Speare & Smith 1992, Speare pers. comm.).

Pathological changes were similar to those encountered in *Bufo marinus* infected with BIV, with renal, hepatic and pulmonary necroses being noted (Speare pers. comm.). Pathological changes also shared similarities with those observed in BIV-infected barramundi *Lates calcarifer* (Moody & Owens 1994) and tilapia *Oreochromis mossambicus* (Ariel 1992). Fish infected with EHNIV displayed similarities in pathology to that observed here in frogs, with necrosis of the kidney, liver and spleen being commonly encountered changes (Langdon et al. 1986, 1988, Langdon & Humphrey 1987, Langdon 1989). Vacuolating encephalopathy of the optic lobes, cerebral hemispheres and thalamus were also noted in EHNIV infections (Langdon et al. 1986, 1988). This was manifested outwardly in neurological behavioural changes in fish. Changes in the brain and spinal cord were noted in tadpoles and metamorphs which were infected with BIV and died. No erratic behavioural changes were observed in the anurans, so these histological findings were possibly due to post-mortem change. Furthermore these neural changes did not stain specifically with immunoperoxidase, suggesting little viral involvement.

Pathological changes in BIV-infected *Limnodynastes terraereginae* and *Litoria latopalmata* were very similar to those observed in spadefoot toads *Scaphiopus*, American toads *Bufo americanus* and Fowler's toads *Bufo woodhousei fowleri* infected with tadpole edema virus (TEV). TEV is an amphibian iridovirus of the genus *Ranavirus* which causes systemic infections of frogs in North America. Pathological changes in the kidney included vascular endothelial necrosis, haemorrhaging into glomeruli which were necrotic, and necrosis of capsules and interstitial tissues in which cells contained pyknotic nuclei. Vascular necrosis of the musculature and submucosa of the stomach, focal haemorrhaging in lungs and evidence of erythrocyte destruction were also encountered (Wolf et al. 1968).

Frog virus 3 (FV3) and Lucké triturus virus (LTV) cause similar pathological changes to those of TEV, but apparently of a lower severity (Granoff 1989). The pathological changes induced by these 3 closely related ranaviruses were similar to those encountered here with BIV.

Iridovirus-like agents isolated from catfish *Ictalurus melas* in France and from sheatfish *Silurus glanis* in Germany also caused systemic diseases resembling those caused by BIV and EHNIV. In *I. melas*, changes included degenerative necrosis of interstitial and tubule cells and destruction of haematopoietic tissues in the kidney, degenerative and necrotising lesions in the spleen, and oedema in the liver (Pozet et al. 1992). Kidneys of *S. glanis* displayed acute necrosis of haematopoietic tissue and degeneration of tubular and collecting duct epithelia, with glomeruli also being affected (Ogawa et al. 1990). Necrosis of splenic haematopoietic tissue, focal hepatic necrosis, and hyperplasia and oedema of gills were also noted (Ogawa et al. 1990). These changes were similar to those observed in Australian iridovirus infections of poikilotherms.

Susceptibility to BIV varied with ontology, as young *Limnodynastes terraereginae* metamorphs were found to be more susceptible than tadpoles. Evidence that BIV caused disease in tadpoles was observed only in 3 individuals at the $10^{2.5}$ TCID₅₀ ml⁻¹ titre viral challenge. Differential susceptibility to viral infection depending on life history stage is not unusual. Mortalities of *Limnodynastes ornatus* due to BIV occurred during or soon after metamorphosis rather than at the tadpole stage (Speare & Smith 1992). Gruia-Gray & Desser (1992) reported that the prevalence of frog erythrocytic virus (FEV), an iridovirus, was 3 times higher in juvenile *Rana catesbeiana* than adults, whilst no infections were encountered in tadpoles. The prevalence of piscine erythrocytic necrosis (PEN) virus, a fish iridovirus, displayed trends resembling those encountered with FEV (Smail 1982).

BIV could be transmitted via natural routes of infection under experimental conditions, with a time lag apparent between infection of the injected and 'in contact' frogs (Table 1). Mortality of the injected frogs took place 6 to 10 d post-challenge, with all frogs in this treatment dying except for the individual which was sacrificed on Day 5 (Table 1). BIV was isolated from the sacrificed individual but no pathological changes were observed, indicating that infection had occurred, but had not progressed to the point where lesions were apparent. Pathological changes occurred in 6 of the 7 frogs which died. The time over which mortality occurred was similar for toads injected with TEV (Wolf et al. 1968). A dose of $10^{7.5}$ ID₅₀ killed *Scaphiopus* between 5 and 8 d, and *Bufo* less than 5 cm in length in 5 to 7 d (Wolf et al. 1968).

The isolation of BIV from frogs and tadpoles showing no pathological changes, and which were sacrificed towards the end of the infection experiments, is indicative that a small proportion of individuals may survive infection and achieve carrier status. Adult frogs and fish may acquire immunity to FEV or PEN respectively following repeated exposure to the viruses (Smail 1982, Gruia-Gray & Desser 1992). Redfin perch *Perca fluviatilis* surviving EHNIV infection probably acquire protective immunity, with some individuals becoming carriers (Langdon & Humphrey 1987, Langdon 1989). One *Bufo* toad sacrificed 37 d after inoculation with TEV was found to have low levels of the virus in the stomach and skeletal muscle, indicating that a subclinical infection had occurred (Wolf et al. 1968). The ability of some infected individuals to become carriers has important implications in the ecology and dynamics of viral transmission. Carrier hosts can be important viral reservoirs in the environment, facilitating continued maintenance and spread of the pathogen. The ability of amphibians to become carriers of viruses potentially able to infect economically important fish species complicates fish health programs, and has important implications for the aquaculture industry.

Amphibian declines are thought to be occurring on a global scale, and causes such as global climatic change and environmental deterioration have been hypothesised (Barinaga 1990, Blaustein & Wake 1990, Ingram 1990, Vitt et al. 1990, Couper 1992, Carey 1993). Among the iridoviruses, erythrocytic viruses have been found in Costa Rica, South Africa and Canada (Desser & Barta 1984, Gruia-Gray et al. 1989, Speare et al. 1991, Gruia-Gray & Desser 1992). FV3, TEV and LTV are considered to be strains of the same virus, belonging to the genus *Ranavirus* (Essani & Granoff 1989). BIV has affinities with the ranaviruses, and is probably a member of this genus (Speare & Smith 1992, Hengstberger et al. 1993). BIV was the first virus reported to be transmissible between amphibians and fish (Moody & Owens 1994). EHNIV is closely related to BIV, and is closely related to the ranaviruses (Hengstberger et al. 1993). The irido-like viruses from sheatfish in Germany (Ogawa et al. 1990) and catfish in France (Pozet et al. 1992) have also been shown to have affinities with the ranaviruses. EHNIV, the sheatfish virus and the catfish virus are similar in size, morphology, polypeptide profiles and antigenic properties, and produce systemic diseases resulting in pathological changes similar to those of BIV and the ranaviruses (Langdon et al. 1986, Langdon & Humphrey 1987, Ogawa et al. 1990, Ariel 1992, Hedrick et al. 1992, Pozet et al. 1992, Moody & Owens 1994). It is possible that other members of the above seemingly related group of viruses might be able to infect both fish and amphibians. Bluegill fry, juvenile brook and rainbow

trout were refractory to TEV (Wolf et al. 1968), but further experimentation is warranted with regards to fish-to-amphibian or vice versa transmission of these viruses. The members of the above group of rana-like viruses have been found around the globe, and it is unknown from where they might have originated or the extent of their geographical range. They produce serious systemic diseases which often result in high mortalities, and some have been shown to have a non-specific host range. The possibility exists that this group of iridoviruses may be involved in the global decline of amphibians.

LITERATURE CITED

- Ariel E (1992) Aspects of Bohle iridovirus in tilapia (*Oreochromis mossambicus*). BSc (Hons) thesis, Department of Biomedical and Tropical Veterinary Sciences, James Cook University, Townsville
- Barinaga M (1990) Where have all the froggies gone? *Science* 247:1033–1034
- Blaustein AR, Wake DB (1990) Declining amphibian populations: a global phenomenon? *TREE* 5:203–204
- Carey C (1993) Hypothesis concerning the disappearance of boreal toads from the mountains of Colorado. *Conserv Biol* 7:355–362
- Couper P (1992) Hope for our missing frogs. *Wildl Aust* 29: 11–12
- Desser SS, Barta JR (1984) An intraerythrocytic virus and rickettsia of frogs from Algonquin Park, Ontario. *Can J Zool* 62:1521–1524
- Eaton BT, Hyatt AD, Hengstberger S (1991) Epizootic haematopoietic necrosis virus: purification and classification. *J Fish Dis* 14:157–169
- Essani K, Granoff A (1989) Properties of amphibian and piscine iridoviruses: a comparison. In: Ahne W, Kurstak E (eds) *Viruses of lower vertebrates*. Springer-Verlag, New York, p 79–85
- Granoff A (1989) Viruses of amphibia: a historical perspective. In: Ahne W, Kurstak E (eds) *Viruses of lower vertebrates*. Springer-Verlag, New York, p 3–12
- Gruia-Gray J, Desser SS (1992) Cytopathological observations and epizootiology of frog erythrocytic virus in bullfrogs (*Rana catesbeiana*). *J Wildl Dis* 28:34–41
- Gruia-Gray J, Petric M, Desser S (1989) Ultrastructural, biochemical and biophysical properties of an erythrocytic virus of frogs from Ontario, Canada. *J Wildl Dis* 25: 497–506
- Hedrick RP, McDowell TS, Ahne W, Torhy C, de Kinkelin P (1992) Properties of three iridovirus-like agents associated with systemic infections in fish. *Dis aquat Org* 13:203–209
- Hengstberger SG, Hyatt AD, Speare R, Couper BEH (1993) Comparison of epizootic haematopoietic necrosis and Bohle iridoviruses, recently isolated Australian iridoviruses. *Dis aquat Org* 15:93–107
- Hyatt AD, Eaton BT, Hengstberger S, Russel G (1991) Epizootic haematopoietic necrosis virus: detection by ELISA, immunohistochemistry and immunoelectron-microscopy. *J Fish Dis* 14:605–617
- Ingram G (1990) The mystery of the disappearing frog. *Wildl Aust* 27:6–7
- Langdon JS (1989) Experimental transmission and pathogenicity of epizootic haematopoietic necrosis virus

- (EHNV) in redbfin perch, *Perca fluviatilis* L., and 11 other teleosts. J Fish Dis 12:295–310
- Langdon JS, Humphrey JD (1987) Epizootic haematopoietic necrosis: a new viral disease in redbfin perch, *Perca fluviatilis* L., in Australia. J Fish Dis 10:289–297
- Langdon JS, Humphrey JD, Williams LM (1988) Outbreaks of an EHN-like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia. J Fish Dis 11:93–96
- Langdon JS, Humphrey JD, Williams LM, Hyatt AD, Westbury HA (1986) First virus isolation from Australian fish: an iridovirus-like pathogen from redbfin perch, *Perca fluviatilis* L. J Fish Dis 9:263–268
- Moody NJG, Owens L (1994) Experimental demonstration of the pathogenicity of a frog virus, Bohle iridovirus, for a fish species, barramundi *Lates calcarifer*. Dis aquat Org 18: 95–102
- Ogawa M, Ahne W, Fischer-Scherl T, Hoffman RW, Schlottfeldt HJ (1990) Pathomorphological alterations in sheatfish fry *Silurus glanis* experimentally infected with an iridovirus-like agent. Dis aquat Org 9:187–191
- Pozet F, Morand M, Moussa A, Torhy C, de Kinkelin P (1992) Isolation and preliminary characterisation of a pathogenic icosahedral deoxyribovirus from the catfish *Ictalurus melas*. Dis aquat Org 14:35–42
- Reed LJ, Muench H (1938) A simple method of estimating fifty percent end points Am J Hyg 27:493–497
- Smail DA (1982) Viral erythrocytic necrosis in fish: a review. Proc R Soc Edinb 81B:169–176
- Speare R, Freeland WJ, Bolton SJ (1991) A possible iridovirus in erythrocytes of *Bufo marinus* in Costa Rica. J Wildl Dis 27:457–482
- Speare R, Smith JR (1992) An iridovirus-like agent isolated from the ornate burrowing frog (*Limnodynastes ornatus*) in northern Australia. Dis aquat Org 14:51–57
- Vitt LJ, Caldwell JP, Wilbur HM, Smith DC (1990) Viewpoint: amphibians as harbingers of decay. BioSci 40:418
- Wolf K, Bullock GL, Dunbar CE, Quimby MC (1968) Tadpole edema virus: a viscerotropic pathogen for anuran amphibians. J infect Dis 118:253–262
- Wolf K, Gravel M, Malsberger RG (1966) Lymphocystis virus: isolation in a centrarchid fish cell line. Science 151: 1004–1005

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